PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 31/70, 45/00, A61N 1/30, 1/00, C12M 1/42

A1

(11) International Publication Number: **WO 00/45823**

(43) International Publication Date: 10 August 2000 (10.08.00)

(21) International Application Number: PCT/US00/02831

(22) International Filing Date: 7 February 2000 (07.02.00)

(30) Priority Data:

60/118,996	8 February 1999 (08.02.99)	US
60/129,189	14 April 1999 (14.04.99)	US

(71) Applicants: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608–2916 (US). EMED CORPORATION [US/US]; 651 Campus Drive, St. Paul, MN 55112 (US).

(72) Inventors: SELBY, Mark; 136 Galewood Circle, San Francisco, CA 94131 (US). GOLDBECK, Cheryl; 725 Camino Ricardo, Moraga, CA 94556 (US). PERTILE, Terry; 3627 Rodeo Drive, Blaine, MN 55449 (US). WALSH, Robert; 17185 Jackson Trail, Lakeville, MN 55144 (US). LIU, Margaret, A.; 3656 Happy Valley Road, Lafayette, CA 94549 (US). ULMER, Jeffery; 160 Summerfield Street, Danville, CA 94506 (US).

(74) Agent: AUERBACH, Jeffery, I.; Liniak, Berenato, Longacre & White, LLC, 6550 Rock Spring Drive, Suite 240, Bethesda, MD 20817 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ELECTRICALLY–MEDIATED ENHANCEMENT OF DNA VACCINE IMMUNITY AND EFFICACY IN VIVO

(57) Abstract

Electrically–mediated delivery technology has been applied to DNA vaccines and substantially higher immune responses have been achieved. In mice and rabbits vaccinated with DNA encoding HIV genes, when administered with constant electric current or constant electric voltage, up to twenty–fold higher immune responses were achieved compared with application of DNA vaccines alone. The increase was achieved under conditions of both constant current (iontophoresis) and constant voltage (electroporation).

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

TITLE OF THE INVENTION

ELECTRICALLY-MEDIATED ENHANCEMENT OF DNA VACCINE IMMUNITY AND EFFICACY IN VIVO

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Provisional Patent Applications Serial Numbers 60/118996 (filed February 7, 1999) and 60/129189 (filed April 14, 1999), both of which applications are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates generally to the use of electrical pulses to enhance DNA vaccine efficacy *in vivo*. More particularly, the present invention relates to the use of electrical pulses to enhance HIV DNA vaccine efficacy *in vivo* and even more particularly to the use of electrical pulses to enhance HIV gag DNA vaccine efficacy *in vivo*.

BACKGROUND OF THE INVENTION

Vaccines composed of live, attenuated pathogens have long been used to provide and/or enhance immunity. Recently, however, the ability to introduce DNA into cells and tissues has led to the proposal that DNA vaccines could be used in lieu of pathogens to provide immunity (for review, see Donnelly *et al.* (1997) *Ann. Rev. Immunol. 15*:617-648).

Although DNA vaccines offer the potential for greater safety, efficacy and protection than that provided by conventional vaccines, the delivery of such DNA to cells has presented several problems (Lai and Bennett (1998) *Crit. Rev. Immunol. 18*:449-484). DNA vaccines given orally have been reported to be

incapable of eliciting an immune response (see Manikan *et al.* (1997) *Crit. Rev. Immunol. 17*:139-154). Likewise, introduction of DNA into the dermis has been found to be complicated both by the susceptibility of the basal cells of the epidermis to transformation, and by rapid turnover of epidermal cells that leads to the expulsion of much of the administered DNA (Lai and Bennett (1998) *Crit. Rev. Immunol. 18*:449-484). Introduction of DNA into muscle cells has been effective to confer immunity in some cases, however, it has been reported that muscle cells do not seem capable of expressing molecules required for efficient antigen presentation (Goebels *et al.* (1992) *J. Immunol. 149*:661-667; Hohfield and Engel (1994) *Immunol. Today 15*:269-274; Michaelis *et al.* (1993) Amer. J. Pathol. 143:1142-1149). Accordingly, a need exists to enhance DNA vaccine efficacy.

The use of electric current has facilitated gene delivery *in vitro* and *in vivo*. Transient discontinuities in the plasma membranes of cells can be induced by short pulses of high-voltage electric current. These discontinuities allow substances, such as DNA to passively enter cells directly into the cytoplasm, thereby avoiding the indirect and inefficient route of endocytosis. As a consequence, more DNA is delivered inside cells and a greater degree of transfection occurs. This process, termed electroporation is widely used for facilitation of transfection of cells *in vitro*.

Recently, the use of electric current to mediate transfer of genes *in vivo* has been reported. Successful transfer of genes has been accomplished for cells of the skin (Titomirov *et al.* (1991) *Biochim. Biophys. Acta 1088*: 131-134; Nomura *et al.* (1996) *J. Immunol. Meth. 193*: 41-49), liver (Heller *et al.* (1996) *FEBS Lett. 389*:225-228; Suzuki *et al.* (1998) *FEBS Lett. 425*: 436-440), tumors (Nishi *et al.* (1996) *Cancer Res. 56*: 1050-1055; Nishi *et al.* (1997) *Hum. Cell 10*: 81-86; Rols *et al.* (1998) *Nature Biotechnol. 16*: 168-171), oviduct (Ochiai *et al.* (1998) *Poult Sci. 77*:299-302), and muscle (Aihara and Miyazaki (1998) *Nature Biotechnol*: *16*: 867-870). In most cases, protein expression was demonstrated, and in some cases biological effects were noted, such as regression of tumors or increased hematocrit after inoculation of erythropoietin DNA (*Rizutto et al.* (1999) *Proc. Natl. Acad.*

Sci. (USA) 96:6417-6422). In one case, induction of an immune response was detected in mice after electroporation *in vivo* with DNA encoding a fusion protein containing a CTL epitope from influenza nucleoprotein (Nomura *et al.* (1996) *J. Immunol. Meth. 193*: 41-49).

A technology related to electroporation, termed iontophoresis, involves the application of an electric field to facilitate movement of charged molecules, such as "naked DNA," in tissue and across biological membranes. Iontophoresis, which involves lower electric current than what is required for electroporation, has been widely used for transdermal delivery of drugs and oligonucleotides.

The efficacy of DNA vaccines in preclinical models has been well documented (for review see Donnelly *et al.* (1997) *Ann. Rev. Immunol. 15*:617-648). The magnitude of immune responses, however, induced in primates is generally lower than that in small animals, and the amount of DNA required for effective immunization of primates is much higher (mg versus μg) (for example, see Kent *et al.* (1998) *J. Virol. 72*:10180-10188; Gramzinski *et al.* (1998) *Molec. Med. 4*:109:118; Richmond *et al.* (1998) *J. Virol. 72*: 9092-9100). In addition, several phase I human clinical studies have been conducted with little or no immune responses reported (Calarota *et al.* (1998) *Lancet 351*: 1320-1325; MacGregor *et al.* (1998) *J. Infect. Dis. 178*:92-100; McClements-Mann *et al.* (1997) Amer. Soc. Virol. Ann. Meet. Abstr. (Vancouver, Canada), p. 115).). In one case, however, cytotoxic T lymphocytes were induced in human volunteers by a malaria DNA vaccine, but no antibodies were detected (Wang *et al.* (1998) *Science 282*:476-480. Therefore, the potency of DNA vaccines must be increased to enable this technology for successful human application. The present invention demonstrates the enhancement of DNA vaccine potency in animals using electrically-mediated delivery of DNA.

SUMMARY OF THE INVENTION

It is a primary object of the invention to provide electrically-mediated enhancement of DNA vaccine efficacy *in vivo*. This object is achieved through the use of electrical current to facilitate gene delivery to cells and tissue. In accordance with an embodiment of the invention, DNA encoding the immunogen of interest is administered parenterally followed by the application of electrical current in either the iontophoresis or electroporation range.

It is a further object of the invention to provide electrically-mediated enhancement of HIV DNA vaccine efficacy *in vivo*. Preferably, the HIV DNA is HIV gag DNA. In embodiments of the invention, such DNA is incorporated into a plasmid and is injected either via an intramuscular (i.m.) or intradermal (i.d.) route.

In detail, the invention provides, a method of enhancing an immune response generated in an animal comprising the steps of:

(A) administering to the animal DNA encoding one or more immunogen of interest; and

(B) applying an electric field to at least the site of such DNA administration.

The invention particularly concerns the embodiment of the above method in which the immunogen is a protein or peptide of a pathogen (especially a bacterium, a fungus, a yeast, a protozoan, or a virus). The invention is particularly concerned with the embodiment of the above method wherein the pathogen is the retrovirus HIV, and wherein the DNA administered in step (A) encodes one or more HIV protein or peptide (especially the HIV gag and/or env proteins or a peptide fragment of either, and most preferably codon-optimized DNA molecules encoding these immunogens).

The invention particularly concerns the embodiment of the above method in which the electrical field is applied under electroporation conditions or under iontophoresis conditions.

The invention additionally provides an apparatus for enhancing an immune response in an animal comprising:

(A) DNA encoding one or more immunogen of interest;

(B) means for administering the DNA to the animal; and

(C) means for applying an electric field to at least the site of such DNA administration.

The invention particularly concerns the embodiment of the above apparatus in which the immunogen is a protein or peptide of a pathogen (especially a bacterium, a fungus, a yeast, a protozoan, or a virus). The invention is particularly concerned with the embodiment of the above apparatus wherein the pathogen is the retrovirus HIV, and wherein the administered DNA encodes one or more HIV protein or peptide (especially the HIV gag and/or env proteins or a peptide fragment of either and most preferably codon-optimized DNA molecules encoding these immunogens).

The invention additionally concerns the embodiment of the above apparatus in which the means for administering the DNA to the animal accomplishes the intramuscular or intradermal administration of the DNA.

The invention additionally concerns the embodiment of the above apparatus in which the electrical field is produced under electroporation or iontophoresis conditions.

The invention additionally concerns the embodiment of the above apparatus in which the means for administering the DNA is a device selected from the group consisting of a single needle probe, a bipolar probe and a combination needle and plate probe.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and **Figure 1B** show the expression of β-galactosidase in mouse muscles that had received β-galactosidase-encoding DNA either without additional treatment (**Figure 1A**) or after electroporation (**Figure 1B**).

Figure 2 shows the ability of electroporation and iontophoresis to enhance the antibody responses of mammals after a single inoculation with DNA encoding the HIV gag protein.

Figure 3 shows the effect of vaccine boosting on antibody responses in mammals inoculated with DNA encoding the HIV gag protein. Note the enhanced immune responses induced by electroporation and iontophoresis even after the booster immunization.

Figure 4 shows the efficacy of electroporation on the anti-HIV gag antibody response of mammals inoculated with a DNA vaccine encoding HIV gag, followed by immunization with recombinant gag protein. Note the enhanced levels of booster response in rabbits that had been primed with DNA and electroporation compared to animals primed with DNA alone.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the enhancement of DNA vaccine efficacy by electrically-mediated administration of the DNA *in vivo*. The recipient of the DNA vaccine may be any mammal (especially a cat, a dog, a horse, a human, a rabbit or a rodent). The invention particularly contemplates that the recipient of the DNA vaccine may be a human.

The DNA vaccine that is administered in accordance with the present invention encodes one or more immunogens. As used herein, an immunogen is a protein or a peptide (i.e., a fragment of a protein) that contains at least one epitope such that the immunogen induces an enhanced immune response in a recipient mammal. As used herein, a treatment or procedure is said to enhance an immune

response if the treatment or procedure increases the extent, duration or degree of the response beyond that observed in the absence of such treatment or procedure. The enhanced immune responses of the present invention include the enhanced production of antibody that is specifically reactive with the immunogen, and the enhanced production of lymphocytes that produce such antibody. An antibody is said to be specifically reactive with an immunogen if it binds to the immunogen in an immunologically relevant manner.

Any of a variety of DNA vaccines may be used in accordance with the present invention include those (for review, see Donnelly *et al.* (1997) *Ann. Rev. Immunol. 15*:617-648; Manikan *et al.* (1997) *Crit. Rev. Immunol. 17*:139-154; Alarcon *et al.* (1999) Adv. Parasitol. 42: 343-410; Lai and Bennett (1998) *Crit. Rev. Immunol. 18*:449-484; Tuteja (1999) *Crit. Rev. Biochem. Molec. Biol. 34*:1-24). In a preferred embodiment, the DNA vaccine of the present invention will encode more than one epitope. Thus, for example, the administered DNA may encode all of the epitopes of a protein associated with HIV (such as the gag or env protein). Alternatively, the administered DNA may encode only a peptide of such protein that contains one (or fewer than all) of the protein's epitopes.

The present invention contemplates that the immunogens encoded by the DNA vaccine of the present invention may comprise a protein or peptide of a pathogen. Such pathogen may be any of a wide group of bacteria (e.g., *E. coli* strains and strains of other enterics (e.g., *Salmonella*), *Clostridria*, *Vibrio*, *Corynebacteria*, *Listeria*, *Nocardia*, *Legionella*, *Bacilli* (especially *B. anthracis*), *Staphylococcus*, *Streptococci* (especially beta-hemolytic *Streptococci* and *S. pneumoniae*), *Borrelia*, *Mycobacterium* (especially *M. tuberculosi*), *Neisseria* (especially *N. gonorrhoeae*), *Trepanoma*, etc.), viruses (e.g., parvoviruses, orthomyxoviruses (especially those causing influenza), paramyxoviruses, picornaviruses (especially rhinoviruses or polioviruses), papoviruses, herpesviruses, togaviruses, retroviruses (especially HIV), rhabdoviruses, etc.), and lower eukaryotes (e.g., fungi, protozoa, yeast, helminths, nematodes, etc. (especially *Dermatophytes*, *Pneumocystis*, *Trypanosoma*, *Plasmodium*, *Candida*,

Cryptococcus, *Histoplasma*, *Coccidioides*, amoeba, schistosomes, etc.). Alternatively, the immunogens of the present invention may encode antigens that are produced by aberrant or diseased cells of the recipient (e.g., cancer cells, etc.), such that the recipient animal will form antibodies that will attack such cells.

The immunogens encoded by the DNA vaccine of the present invention may be related to one another, may be clinically related, or may be unrelated to one another. As used herein, immunogens are related to one another if the immune responses that they induce elicit antibodies that bind to the same cell, microbe, virus, etc. For example, DNA that encodes epitopes of the gag or env protein would encode related immunogens. Immunogens are said to be clinically related to one another if the immune responses that they induce elicit antibodies that bind to different cells, microbes, viruses, etc. that are associated with the same clinical condition. For example, individuals suffering from Acquired Immunodeficiency Syndrome (AIDS) may develop infections caused by the bacterium *Listeria monocytogenes*, and by the yeast *Candida*. DNA that encodes epitopes of a *Listeria monocytogenes* protein and a *Candida* protein would encode clinically related immunogens. Alternatively, the DNA vaccine of the present invention may encode an epitope of a poliovirus and an epitope of a measles virus, and thus provide unrelated immunogens.

Most preferably, the DNA of the DNA vaccine of the present invention will contain regulatory elements (promoters, translation initiation sites, etc.) operably linked to the immunogen-encoding sequences and sufficient to permit the protein expression of the immunogen. Alternatively, the administered DNA will not contain such regulatory elements, and will require cellular processes (such as recombination or integration into nuclear or mitochondrial DNA, etc.) in order to produce the encoded immunogen.

The DNA vaccine of the present invention may comprise more than one molecular species of DNA. Such multiples species may contain the same DNA sequence (e.g., a mixture of circular and linearized plasmids), or may contain

different DNA sequences encoding the same immunogen (e.g., a mixture of DNA molecules of different length all of which contain a particular immunogen-encoding sequence), or may contain DNA sequences encoding different immunogens. The administered DNA can be either "naked" DNA (i.e., free of associated protein or lipids), or may be complexed with protein or lipids or other molecules. For example, the DNA can be administered with a local anesthetic such as bupivicaine or a myotoxin such as cardiotoxin, or with proteins that assist in the efficient presentation of antigen (e.g., CD80, CD86, etc.) (Tuteja (1999) *Crit. Rev. Biochem. Molec. Biol. 34*:1-24). The DNA may encode only the desired immunogen or immunogens, or may encode other additional proteins or peptides that may be linked or unlinked to the immunogen and that enhance immunogen stability or immunogenicity. The DNA may also encode protein extraneous to the immunogenicity of the immunogen that is encoded by the DNA; such extraneous protein may likewise be linked or unlinked to the immunogen. The DNA of the DNA vaccine of the present invention may contain untranslated or untranscribed DNA.

The DNA can be incorporated into a recombinant expression vector such as a chimeric virus, a plasmid DNA, etc. The DNA is preferably dissolved or suspended in a buffer or other solution (e.g., 5% dextrose).

In a particularly preferred embodiment, DNA, preferably in the form of plasmid DNA, is administered (especially by injection) into tissue and voltage pulses are applied between electrodes disposed in the tissue, thus applying electric fields to cells of the tissue. The electrically-mediated enhancement covers administration using either iontophoresis or electroporation *in vivo*. Suitable techniques of electroporation and iontophoresis are provided by Singh *et al.* (1989) *Drug Des. Deliv. 4*:1-12; Theiss U *et al.* (1991) *Methods Find. Exp. Clin. Pharmacol. 13*:353-359; Singh and Maibach (1993) *Dermatology. 187*:235-238; Singh and Maibach (1994) *Crit. Rev. Ther. Drug Carrier Syst. 11*:161-213; Su *et al.* (1994) *J. Pharm. Sci. 83*:12-17; Costello *et al.* (1995) *Phys. Ther. 75*:554-563; Howard *et al.* (1995) *Arch. Phys. Med. Rehabil. 76*:463-466; Kassan *et al.* (1996)

J. Amer. Acad. Dermatol. 34:657-666; Riviere *et al.* (1997) *Pharm. Res. 14*:687-697; Zempsky *et al.* (1998) *Amer. J. Anesthesiol. 25*:158-162; Muramatsu *et al.* (1998) *Int. J. Mol. Med. 1*:55-62; Garrison J. (1998) *Med. Device Technol. 9*:32-36; Banga *et al.* (1998) *Trends Biotechnol. 16*:408-412; Banga *et al.* (1999) *Int. J. Pharm. 179*:1-19; Singh *et al.* (1999) *Anticancer Drugs. 10*:139-146; Neumann *et al.* (1999) *Bioelectrochem. Bioenerg. 48*:3-16; and Heiser (2000) *Methods Mol. Biol. 130:*117-134. Whereas any suitable route of inoculation may be employed, of intra-muscular (i.m.), intra-dermal (i.d.), or sub-cutaneous (s.c.), i.m. injection is the most efficacious. Enhanced immune responses are, however, also seen after i.d. injections.

The nature of the electric field generated in accordance with the present invention is determined by the nature of the tissue, the size of the selected tissue and its location. It is desirable that the field be as homogeneous as possible and of the correct amplitude. The use of insufficient or excessive field strength is to be avoided. As used herein, a field strength is excessive if it results in the lysing of cells. A field strength is insufficient if it results in a reduction of efficacy of 90% relative to the maximum efficacy obtainable. The electrodes may be mounted and manipulated in many ways known in the art.

The waveform of the electrical signal provided by the pulse generator can be an exponentially decaying pulse, a square pulse, a unipolar oscillating pulse train or a bipolar oscillating pulse train. The waveform, electric field strength and pulse duration are dependent upon the type of cells and the DNA that are to enter the cells via electrical-mediated delivery and thus are determined by those skilled in the art in consideration of these criteria.

Any number of known devices may be used for delivering the DNA vaccine and generating the desired electric field. Examples of suitable devices include, but are not limited to, a single needle probe, a bipolar probe and a combination needle and plate probe. The single needle probe exemplified herein is a single stainless steel needle, with an insulation stop that provides preferably about 3mm of active

zone. The single needle serves as the negative electrode and the plasmid delivery device. The positive electrode is a hypodermic needle located in the opposite leg or arm of the recipient patient or test animal. The bipolar probe exemplified herein contains two stainless steel needles preferably about 3mm in length and separated by a distance of preferably about 0.4cm. One needle carries a positive charge and one needle carries a negative charge. The combination needle and plate probe exemplified herein contains two stainless steel needles preferably about 3 mm in length and separated by a distance of preferably about 0.4cm. The needles are insulated except for the distal 1mm. Both needles serve as the negative electrodes. The needles protrude from a stainless steel block. The block sits on the surface of the skin and serves as the positive electrode. The separation distance between the nearest active area on the block to the nearest active area on the needles is preferably about 2.5mm. The needles are insulated from direct contact with the stainless steel block.

Preferred electrical field conditions for i.m. administration are as follows: 50mA for 10msec for 5 pulses then rotated 90° (i.e., orthogonally) for 5 additional pulses; 120V for 10 msec for 5 pulses then rotate orthogonally for 5 additional pulses when using the bipolar probe; and 80V for 10msec for 5 pulsed then rotate orthogonally for 5 additional pulses when using the combination plate and needle probe. Preferred electrical field conditions for i.d. administration are as follows: 50mA for 50msec for 5 pulses then rotate orthogonally for 5 additional pulses; and 120 V for 50 msec for 5 pulses then rotate orthogonally for 5 additional pulses when using the bipolar probe.

Preparations of DNA for parenteral administration include but are not limited to sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate.

The increased DNA vaccine potency observed after iontophoresis or electroporation may reflect a facilitation, by the electric current, of the distribution

of DNA within the injected tissue and/or uptake of DNA by cells, leading to increased transfection. The ensuing increase in the amount of antigen expressed by cells is likely to have played a role in the elevated immune responses. Alternatively, or in addition, infiltration of inflammatory cells (in response to the electric current) could have an "adjuvant" effect on the produced antigen. The present invention demonstrates that DNA vaccine potency can be increased by application of electric current. The results indicate that a significant limitation to efficient transfection of cells *in vivo* by naked DNA vaccines in the past (possibly accounting for the lack of efficacy of DNA vaccines in larger animals, such as primates, in the past) has been the distribution of the introduced DNA within tissue and/or uptake of DNA by cells. Iontophoresis and electroporation (as well as equivalent means for facilitating the delivery of DNA into cells and tissue can be used to surmount this problem and enable the development of DNA vaccines.

Having now fully described the invention, the same will be further illustrated by way of the following examples, which are meant solely to illustrate the invention and are not to be construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention.

Example 1
Materials And Methods For *In Vivo* Electrical-Enhanced Delivery Of DNA.

Bacterial Strain and plasmid preparation

The bacteria Escherichia coli strain HB101 were transformed with the plasmids pCMV HIV gag prepared as described in U.S. Provisional Patent Application 60/114495, filed 31 December 1998, or pCMV KM LUC encoding firefly luciferase reporter gene (LUC). In brief, a luciferase expression plasmid was obtained from Promega Corporation (Madison, WI). *E. coli* strain XL-1 Blue (Stratagene, La Jolla), carrying the expression plasmid, was grown in LB; antibiotic selection employed 50 μg/ml of ampicillin. Plasmids were purified using Qiagen Endo Free Plasmid Maxi Kits (Qiagen, Inc., Chatsworth, CA) according to the

manufacturer instructions and resuspended in 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL).

The plasmid pCMV HIV gag was used as a source of gag-encoding DNA. The plasmid expresses high levels of HIV-1 gag, due to a potent CMV promoter with intron A and a codon-optimized gag encoding region (see U.S. Provisional Patent Application Serial No. 60/168,471, filed December 1, 1999). The plasmid was grown in *E. coli* strain HB101, purified using a Qiagen Endofree Plasmid Giga kit, (Qiagen, Inc.) and resuspended in 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL). Plasmid concentrations were analyzed by measuring absorbance at 260 nm.

Expression of the encoded antigens was verified by transient expression studies in B16 cells. One μg of each plasmid DNA was used for Lipofectin (Gibco/BRL) transfection following the manufacturers protocol; $5x10^5$ cells were used per 3 cm tissue culture dish; incubation time for DNA/Lipofectin on cells was for 4 hours. Supernatants were harvested 36 hours after removal of the DNA/Lipofectin solution and cells were lysed in 500 μl phosphate buffered saline (PBS)/0.5% TritonX100 (Mallinckrodt). Luciferase activity in cell lysates was detected by commercial Luciferase Reporter Gene Assay (Roche, Indianapolis, IN).

Immunization Procedure:

Female 6-8 week old CB6F1 or BalbC mice (Charles River) were anesthetized using 4 parts ketamine HCl, 100mg/ml stock solution, (Fort Dodge Animal Health, Fort Dodge, Iowa) 1 part xylazine, 20mg/ml, (LLoyd Labs, Shenandoah, Iowa). The mice received 1μl per gm of body weight intramuscularly in the posterior thigh. The anterior tibialis (TA) muscle was shaved and the animals were injected with 10 μg of plasmid in a volume of 50 μl. To control needle depth, the syringe was covered with polyethylene tubing (i.d. 0.38) to expose only the bevel. The animals were injected intramuscularly, intradermally or subcutaneously. For each of the types of injections, an electrical field was then applied to the animals except to the control group of animals. One group of

animals received an electrical field in the iontophoresis range. That is, using a single needle probe set-up 50 mA at a 10 msec pulse width, 1 Hz frequency for a total of 60 pulses were delivered. Another group of animals received an electrical field in the low electroporation range. That is, 40 V at 10 msec pulse width, 1 Hz frequency for 5 pulses were delivered plus 5 additional pulses were delivered after the probe was turned in an orthogonal direction to the first set of 5 pulses. Another group of animals received an electrical field in the high electroporation range. That is 80 V, at a 10 msec pulse width, 1 Hz frequency for 5 pulses were delivered plus 5 additional pulses were delivered after the probe was turned in an orthogonal directed to the first set of 5 pulses. Serum samples were collected at 2, 4, 8 and 12 week intervals and analyzed by the below-outlined procedures. The results of this experiment showed enhanced antibody titers in the animals inoculated by the i.m. route with enhancement ranging from 8- to 20-fold.

Immunoassays:

The mouse anti-p55 IgG antibodies were measured by one of two methods, chemi-luminescent or colormetric ELISA assays.

Chemi-luminescent ELISA

MicroLite 2, 96 well flat bottom plates (Dynes Technologies, Chantilly, VA) were coated with HIV p24 protein at 5µg/ml in 10mM tris pH=7.5, 50 µl per well and incubated at 4°C overnight. The plates were washed 3X with wash buffer [1X AquaLite® Wash Buffer (SeaLite Sciences, Inc. Bogart, GA) containing 0.3% Tween 20 (Sigma, St. Louis, MO)], and blocked at 37°C for 1 hour with 150 µl/well blocking buffer [1X Streptavidin AquaLite® Assay buffer (SeaLite Sciences, Inc. Bogart, GA) containing 5% goal serum]. The plates were washed 3X and the test sera were diluted 1/300 or 1/9000 followed by serial 3-fold dilutions in the blocking buffer. A volume of 50 µl of each dilution was added per well and the plates were incubated at 37°C for 1 hour. The plates were washed 6X and incubated for 1 hour at 37°C with 50 µl/well of Goat anti-mouse IgG –Biotin (Sigma St. Louis, MO), diluted 1/1000 in block buffer. After washing 6X, the plates were incubated at 37°C for 1 hour with Streptavidin-Aqualite® (SeaLite

Sciences, Inc. Bogart, GA), diluted 1/500 in wash buffer, 50 µl/well. The plates were washed 6X and stored in wash buffer until reactivity was measured on the luminometer (MLX, Dynex Technologies, Chantilly, VA). Setting for the luminometer – mode: Integrate Flash, Gain: High, Data: Table, Delay window: 0.00 sec., Integrate window: 3.00 sec., Before peak: 0.10 sec., After peak: 2.00 sec, calibrate on each well. The plates were tapped dry and put into the luminometer. Fifty microliters of 1X AquaLite® Trigger Buffer (SeaLite Sciences, Inc. Bogart, GA) were automatically dispensed per well and the relative light units (RLU) measured. Endpoint titers were calculated as the inverse of the dilution that yields an RLU equal to the background plus 5 times the standard deviation.

Colormetric ELISA

Wells of Immulon 2 HB "U" bottom microtiter plates (Dynex Technologies, Chantilly, VA) were coated with HIV p55 protein at 5 µl/ml in PBS, 50 µl per well, and incubated at 4°C overnight. The plates were washed 6X with wash buffer [PBS, 0.1% tween (Sigma, St. Louis, MO)] and blocked at 37°C for 1 hour with 150µl/well blocking buffer [PBS, 0.1% tween 20 (Sigma, St. Louis MO), 1% goat serum]. Test sera were diluted 1/25 followed by serious 3-fold dilutions in blocking buffer. The block solution was aspirated the plates were incubated at 37° for 2 hours with 150µl/well of Goat anti-mouse IgG-HRP (Caltag, Burlingame, CA) diluted 1/40,000 in block buffer. Following a final 6 washes, the plates were developed with OPD for 30 min. The OPD developer consists of 1 tablet (10 mg) o-phenylenediamine, 12 ml buffer (0.1M citric acid, 0.1M dibasic sodium phosphate), 5µl 30% H_2O_2. The reaction was stopped with 50µl per well 4H H_2SO_4 and optical density was measured at dual wavelengths 492-690. The reported titers correspond to the reciprocal of the serum dilution producing an absorbance value of 1.0.

Example 2
Enhancement Of Luciferase Gene Expression In Muscle Cells In Mammals

Previous reports have demonstrated that application of electric current after injection of plasmid DNA has resulted in increased expression of the encoded proteins in the injected tissues (for example see Mir *et al.* (1999) *CR Acad. Sci. III 321*:893-899; Mathieson (1999) *Gene Ther. 6*: 508-514). In order to demonstrate the ability of electoporation and iontophoresis to facilitate the distribution and/or uptake of DNA into mammalian cells and tissue, mice were injected with DNA encoding the readily discernable marker enzyme luciferase (Luc).

Immunization Procedure

Female 6-8 week old CB6F1 mice (Charles River) were anesthetized using 4 parts ketamine HCl, 100 mg/ml stock solution (Fort Dodge Animal Health, Fort Dodge, Iowa), 1 part xylazine, 20 mg/ml (Lloyd Labs, Shenandoah, Iowa). The mice received 1 µl per mg of body weight intramuscularly in the posterior thigh. The tibialis anterior (TA) muscle was shaved and the animals were injected with 10 µg of plasmid in a volume of 50 µl. To control needle depth, a 0.3 cc insulin syringe was covered with polyethylene tubing (i.d. 0.38) to expose only the bevel.

In some instances, electric current was applied to the injected muscles as follows. For constant current deliveries (iontophoresis), plasmid DNA in 5% dextrose was injected into the right tibialis anterior muscle using a single needle delivery probe, which has a functional length of 3 mm. Following plasmid injection, the plasmid delivery needle was attached to the negative lead from the controller and a needle electrode placed in the contralateral leg was attached to the positive lead. Constant current pulses of 5 mA in amplitude, 10 msec in width, were given at a frequency of 1 Hz for 1 min. For constant voltage deliveries (electroporation), plasmid DNA in PBS was injected into the right tibialis anterior muscle as previously described. Electrical energy delivery was performed through a bipolar needle probe that was placed over the site of plasmid injection. The probe needles had a separation distance of 0.4 cm and a needle length of 0.3 cm.

The probe was connected to a constant voltage power supply and 5 constant voltage pulses, 50 msec in width, either 100 or 200 V cm-1, were applied in one orientation, the probe was rotated 90 degrees and 5 additional pulses were applied.

Measurement of Luciferase Activity

Mice were sacrificed up to 14 days post vaccination, and TA muscles were collected and flash frozen in liquid nitrogen. The frozen tissue was homogenized with a mortar and pestle (on dry ice), lysed with 0.5 ml 1X reagent lysis buffer (Promega, Madison, WI), and vortexed for 15 minutes at room temperature. The samples were subjected to 3 freeze thaws and centrifuged for 10 minutes at 10,000 X g. Supernatants were collected and stored at -80°C until assayed. The ML3000 microplate luminometer (Dynex Technologies, Chantilly, VA) measured the luciferase activity by automatically dispensing 100 µl of luciferase assay reagent (Promega, Madison, WI) into wells containing 20 µl of supernatant, and measuring the relative light units (RLU). The setting for the luminometer were the following, Mode: enhanced flash, Gain: medium, Delay time: 1 sec., Integrate time: 5 sec., calibrate each run. Sample values were extrapolated from a standard curve prepared from QuantiLum® Recombinant Luciferase (Promega, Madison, WI). Results are expressed as ng luciferase per mg muscle protein, with protein determination by BCA Protein Assay Reagent (Pierce).

The results of this experiment are shown in **Table 1**, and indicate that electoporation and iontophoresis facilitated the distribution and/or uptake of DNA into mammalian cells and tissue. In **Table 1**, results are expressed as ng luciferase activity per mg muscle protein. Numbers in parentheses indicate standard deviation of the mean (sd).

Table 1			
Luc DNA Treatment	**Luc Activity**	**Mean (sd)**	**Fold Increase**
(control) (10 µg)	6.76 0.74 0.44 9.11 1.92	3.794 (3.91)	1.00
Ionto (10 µg)	26.63 10.54 23.46 5.51 20.61	17.35 (8.95)	4.57
Electro (10 µg)	18.5 35.02 39.02 33.22 13.06	27.764 (11.30)	7.32

Example 3
Enhancement Of Luciferase Gene Expression
In Muscle Cells In Mammals

In order to assess the duration of luciferase gene expression in mammalian tissue, groups of 6 CB6 F1 mice were inoculated with 10 µg of luciferase (Luc) DNA in the TA muscle of one leg. One group of mice was not further treated and one group was treated with electroporation (Electro). At 4 and 14 days after inoculation, the muscles were collected and luciferase activity was measured and expressed as ng luciferase activity per mg muscle protein. The data (**Table 2**) showed a significant enhancement of luciferase gene expression in mammalian tissue that had been subjected to electroporation, relative to non-electroporated, control animals. In **Table 2**, numbers in brackets indicate standard deviation of the mean (sd).

Table 2				
Luc DNA Treatment	4 Days		14 Days	
	Luc activity	Mean (sd)	Luc activity	Mean (sd)
(control) (25 µg)	0 5.02 0.06 0.17 0.05 0	1.32 (2.03)	0 0 0 0 0 0	0 (0)
(Electro) (10 µg)	17.3 42.9 9.38 69.7 92.8 72.9	50.8 (33.2)	5.26 3.63 18.9 7.52 40.4 0.71	12.7 (14.9)

Example 4
Enhancement Of β-Galactosidase Gene Expression In Muscle Cells In Mammals

To further demonstrate the ability of electoporation and iontophoresis to facilitate the distribution and/or uptake of DNA into mammalian cells and tissue, mice were injected with DNA encoding a different readily discernable marker enzyme (β-galactosidase).

CB6 F1 mice were inoculated with 100 µg of pCMV β-gal, a β-galactosidase-encoding DNA, in the TA muscle of one leg. The plasmid uses the same promoter as that used for HIV gag and env to express β-galactosidase. One group of mice was not further treated, one group was treated with electroporation, and another with iontophoresis. At 1 day after inoculation, the muscles were collected and prepared for microscopy (magnification = X). The data (**Figure 1A** (untreated); **Figure 1B** (electroporation)) indicated that electroporation had substantially facilitated the distribution and/or uptake of DNA into mammalian cells and tissue. A similar result was observed in mouse tissue that had been subjected to iontophoresis.

Thus, DNA plasmids encoding the reporter genes luciferase and β-galactosidase were employed to measure transfection of muscles cells *in vivo*. At 4 and 14 days after a single inoculation of DNA, luciferase expression was found to be higher in muscles treated with electric current (as compared to untreated muscles (see **Example 2, Table 1**)). This was true for muscles that had been subjected to both iontophoresis (4.6-fold) and electroporation (7.3-fold). Similarly, the number of muscle fibers detectably transfected after inoculation of β-galactosidase DNA was found to have been substantially increased by iontophoresis and electroporation, as compared to untreated muscles, as judged by β-galactosidase staining of muscle tissue sections. In addition, as noted previously (Mir *et al.* (1999) *CR Acad. Sci. III 321*:893-899), application of electric current appears to decrease the variability of reporter gene expression in muscle cells. Therefore, application of electric current facilitates delivery of DNA to muscle cells *in situ* promotes efficient transfection.

Example 5
Enhancement Of Antibody Responses In Mammals

In order to demonstrate the ability of electroporation and iontophoresis to enhance the antibody responses of mammals, groups of 4-6 CB6 F1 mice were inoculated a single time with 10 μg of DNA encoding the HIV gag protein.

The plasmid pCMV HIV p55 gag, grown in E. coli strain HB101, as described above, was employed as the source of the gag-encoding DNA. The DNA was inoculated into the TA muscle of one leg. One group of mice was not further treated, one group was treated with iontophoresis and another with electroporation. Sera from mice were analyzed for anti-gag antibody titer at 2, 4, 8 and 12 weeks after inoculation. The data are shown in **Figure 2**. In **Figure 2**, data are plotted as geometric mean ELISA titer and error bars indicate SEM. At all time points tested, antibody titers in mice that had been subjected to iontophoresis and electroporation were 8- to 20-fold higher than in animals receiving no further treatment (**Figure 2**). As with luciferase expression levels, in general, electroporation conditions

appeared slightly superior to iontophoresis for enhancement of antibody responses. The data indicate a pronounced enhancement of antibody response in animals subjected to electroporation and iontophoresis, relative to the response of control animals.

Example 6
Effect Of Vaccine Boosting On Antibody Responses In Mammals

In order to demonstrate the effect of vaccine boosting on antibody responses in mammals, groups of 6 CB6 F1 mice were inoculated with 10 μg of DNA encoding the HIV gag protein. Inoculation was into the TA muscle of one leg of the animals at 3 and 6 weeks. One group of mice was not further treated (**Figure 3**, open bars), one group was treated with iontophoresis (**Figure 3**, solid bars) and another with electroporation (**Figure 3**, shaded bars). Sera were collected at 3 weeks after each immunization and analyzed for antibody responses. Data are plotted as geometric mean ELISA titer and error bars indicate SEM. Antibody titers were elevated in all groups after the booster injection, but the approximately 10-fold enhancement in titers observed in mice receiving electric current was maintained even after the boost (**Figure 3**).

Example 7
Effect Of Conditions Of Iontophoresis And Electrophoresis On Antibody Responses In Mammals

In order to demonstrate the effect of the conditions of iontophoresis and electroporation on mammalian antibody responses, groups of 6 CB6 F1 mice were inoculated with 10 μg of HIV gag DNA (obtained as described above) in the TA muscle of one leg at 3 weeks. Groups of mice were treated as indicated in **Table 3**. Sera were collected at 3 weeks and analyzed for antibody responses. In **Table 3**, data are tabulated as geometric mean ELISA titer and as fold increase over titers achieved in vaccinated but untreated mice. The results show that enhancement of DNA vaccine potency is achieved across a wide range of conditions.

Table 3					
Treatment	**Conditions**	**Number of Pulses**	**Duration (msec)**	**Geometric Mean Titer**	**Fold Increase**
DNA control	-	-	-	414	1
Ionto	50 mA	60	10	1071	2.59
Ionto	50 mA	10	10	2521	6.09
Ionto	50 mA	10	50	1738	4.20
Ionto	100 mA	10	10	1876	4.53
Ionto	100 mA	10	50	2293	5.54
Electro	25 V/cm	10	10	479	1.16
Electro	50 V/cm	10	10	1099	2.65
Electro	50 V/cm	10	50	2390	5.77
Electro	100 V/cm	10	50	1800	4.35
Electro	200 V/cm	10	10	2208	5.33
Electro	200 V/cm	10	50	2079	5.02
Electro	300 V/cm	10	10	2834	6.85
Electro	400 V/cm	10	10	1359	3.28

Example 8
Efficacy Of Intradermal Administration
Of Iontophoresis And Electroporation In Mammals.

In order to assess the efficacy of intradermal administration of iontophoresis and electroporation in mammals, groups of 6 CB6 F1 mice were inoculated with 10 μg of HIV gag DNA intradermally on the backs. One group of mice was not further treated (DNA control), one group was treated with iontophoresis and another with electroporation at the conditions indicated in **Table 4**. Sera were collected at 3 weeks after immunization and analyzed for antibody responses. In **Table 4**, data are tabulated as geometric mean ELISA titer and fold increase over titers achieved in vaccinated but untreated mice. As shown, electroporation and iontophoresis are also effective for the intradermal route of administration of DNA vaccines.

Table 4					
Treatment	**Conditions**	**Number of Pulses**	**Duration (msec)**	**Geometric Mean**	**Fold Increase**
DNA control	-	-	-	472	1
Ionto	50 mA	60	10	696	1.47
Ionto	50 mA	10	10	1291	2.74
Ionto	50 mA	10	50	626	1.33
Ionto	100 mA	10	10	2376	5.03
Ionto	100 mA	10	50	1134	2.40
Electro	150 V/cm	10	10	2768	5.86
Electro	300 V/cm	10	10	851	1.80
Electro	450 V/cm	10	10	132	0.28
Electro	600 V/cm	10	10	887	1.88
Electro	750 V/cm	10	10	480	1.02
Electro	75 V/cm	10	50	224	0.47
Electro	150 V/cm	10	50	728	1.54
Electro	225 V/cm	10	50	2202	4.67
Electro	300 V/cm	10	50	6125	12.98
Electro	375 V/cm	10	50	937	1.99

Example 9
Efficacy Of Plate Electrode For Iontophoresis And Electroporation In Mammals

In order to demonstrate the efficacy of employing a plate electrode for iontophoresis and electroporation in mammals, groups of 6 CB6 F1 mice were inoculated with 10 μg of HIV gag DNA in the TA muscle of one leg. Groups of mice were treated as indicated in **Table 5**. The combination needle and plate electrode system consists of 3 electrically conducting components, plus electrical leads for connections, and a holder apparatus. Two of the electrically conductive components represent needle electrodes, of the same polarity (typically negative). These needle electrodes are fabricated of stainless steel (cylindrical, grade 316). Needle lengths were 3mm. The needles were encapsulated within insulation, and were retained in the electrode assembly, surrounded by the plate electrode. The plate electrode consisted of a stainless steel block, with dimensions of 1 x 1 x 1 cm. The needle electrodes extended through the plate electrode, with approximately 3 mm length extending beyond the surface of the electrode. Insulation around the needle prevented passage of electric current from the needle

directly to the plate electrode. For in vivo application, the electric current path was from the power source through the connector cable to the needle electrodes. Electric current was then transmitted from the end of the needle electrodes through biological tissue, to the plate electrode, and thus through a connecting cable to the power source, completing the circuit. The shortest electrically conductive path through tissue is approximately 2.5 mm. This is accounted for by the 2 mm of insulated needle electrode extending above the plate electrode, and the diameter of the holes through the plate electrode, through which the needle electrodes extend.

The electrode assembly was used to deliver a series of electrical energy pulses in either constant voltage (electroporation) or a constant current (iontophoresis) mode. Sera were collected at 6 weeks and analyzed for antibody responses. One group of mice was not further treated (DNA control). Other groups were treated with iontophoresis and electroporation at the indicated conditions. In **Table 5**, data are tabulated as geometric mean ELISA titer and fold increase over titers achieved in vaccinated but untreated mice. The results indicate that a significant increase in antibody titer could be obtained using the needle and plate electrode system to deliver current for electroporation or iontophoresis.

Table 5

Treatment	Conditions	Number of Pulses	Duration (msec)	Geometric Mean	Fold Increase
DNA control	-	-	-	198	1
Ionto	50 mA	10	10	1596	8.06
Ionto	100 mA	10	10	1235	6.24
Electro	200 V/cm	10	10	1411	7.13
Electro	300 V/cm	10	10	1252	6.32

Example 10
Efficacy Of Electroporation On Anti-HIV Gag Antibody Responses in Mammals

In order to demonstrate the efficacy of electroporation on the anti-HIV gag antibody response of mammals, groups of 4-6 New Zealand white rabbits were inoculated with a combination DNA vaccine consisting of 500 μg of DNA encoding the HIV gag protein and 1 mg of DNA encoding the HIV env protein.

The plasmid pCMV HIV gag was used as the source of the gag- encoding DNA. Plasmid pCMV HIV env was employed as the source of the env-encoding DNA. The plasmid expresses high levels of HIV-1 env, due to a potent CMV promoter with intron A and a codon-optimized env-encoding region (see U.S. Provisional Patent Application Serial No. 60/168,471, filed December 1, 1999).

Inoculations were into the hind leg gracilis muscles at 0, 6 and 12 weeks. One group of rabbits received DNA without further treatment (DNA control). Other groups were treated with electroporation with a 6-needle electrode or a 2-needle electrode. The two-needle array electrodes (BTX) were inserted into the muscle immediately after DNA delivery for electroporation. The distance between the electrodes was 5 mm and the array was inserted longitudinally relative to the muscle fibers. *In vivo* electroporation parameters were: 20V/mm distance between the electrodes, 50 msec pulse length, 6 pulses with reversal of polarity after three pulses, at 1 pulse per second, given by a BTX 820 square wave generator. The electroporation with a 6-needle electrode array formed a circle (Genetronics, Inc.). The diameter of the electrode array was 1 cm, with a needle length of 1 cm. Six electroporation pulses of 20V/mm, 50 msec pulse length, one pulse per second were given by a BTX 820 square wave generator, combined with an electronic switch (Genetronics, Inc.) to rotate the electric field in 60 degree increments after each discharge (Hofmann *et al.* (1996) *IEEE Engineer. Med. Biol. 15*:124-132).

At 26 weeks, all rabbits were boosted with recombinant gag protein and sera were collected at 2 weeks post-protein boost and analyzed for anti-gag antibody responses. Anti-HIV gag antibodies were measured by ELISA as follows. Wells of Immulon 2 HB "U" bottom microtiter plates (Dynex Technologies, Chantilly, VA) were coated with HIV p55 protein at 5μg/ml in PBS, 50 μl per well, and incubated at 4°C overnight. The plates were washed 6X with wash buffer [PBS, 0.1% Tween 20 (Sigma, St. Louis, MO)] and blocked at 37°C for 1 hour with 150μl/well blocking buffer [PBS, 0.5% casein, and 5% goat serum]; the dilution buffer was blocking buffer plus 0.3% Tween 20. The secondary antibody was goat anti-rabbit IgG used at 1/20,000; and the OD cutoff used was 0.6. Test

sera were diluted 1/25 followed by serial 3-fold dilutions in blocking buffer. The blocking buffer was aspirated and the plates were incubated at 37°C for 2 hours with 50µl/well of each dilution. After washing 6 times, the plates were incubated for 1 hour at 37°C with 50µl/well of Goat anti-mouse IgG-HRP (Caltag, Burlingame, CA) diluted 1/40,000 in blocking buffer. Following a final 6 washes, the plates were developed with OPD for 30min. The OPD developer consists of 1 tablet (10 mg) o-phenylenediamine, 12 ml buffer (0.1M citric acid, 0.1M dibasic sodium phosphate), 5µl 30% H_2O_2. The reaction was stopped with 50µl per well 4N H_2SO_4 and optical density was measured at dual wavelengths 492-690. The reported titers correspond to the reciprocal of the serum dilution producing an absorbance value of 1.0.

For measurement of anti-env antibodies in rabbits and guinea pigs, Nunc Immunoplate U96 Maxisorp plates (Nalge Nunc International, Rochester, NY) were coated with 200ng per well of recombinant gp120SF2 protein and incubated for at least 14 hours at 4°C. Between steps, the plates were washed in a buffer containing 137mM NaCl and 0.05% Triton X100. Serum samples were initially diluted 1:25 or 1:100 (in a buffer containing 100mM $NaPO_4$, 0.1% Casein, 1mM EDTA, 1% Triton X-100, 0.5M NaCl and 0.01% Thimerosal, pH 7.5) and were serially diluted 3-fold. The plates were incubated for 50 minutes. After washing in a buffer containing 137mM NaCl, 0.05% Triton X-100, the samples were then reacted with an HRP-conjugated second antibody. The plates were then developed using a TMB substrate kit (Pierce, Rockford, IL). The plates were stopped with either 2N H_2SO_4 or 10% SDS, respectively and read at wavelengths of 450nm or 415nm, respectively. Anti-env antibody responses were measured as the dilution at which an OD of 0.6 was achieved.

The data is shown in **Figure 4**, and indicates that electroporation was effective in enhancing the induced immune response. In **Figure 4**, data are plotted as geometric mean ELISA titer and error bars indicate SEM.

Example 11
Efficacy Of Electroporation On Anti-HIV Env Antibody Responses in Mammals

As a further demonstration of the efficacy of electroporation on the antibody response of mammals, groups of 4 New Zealand white rabbits were inoculated with a combination DNA vaccine consisting of 500 μg of HIV gag-encoding DNA and 1 mg of HIV env-encoding DNA (obtained as described above) in the hind leg muscles at 0 and 6 weeks. One group of rabbits received DNA without further treatment and one group was treated with electroporation with a 6-needle electrode as described above. Sera were collected at 2 weeks post the second DNA immunization and analyzed for anti-env antibody responses. The data are shown in **Table 6**. In **Table 6**, data are tabulated as individual ELISA titers, geometric mean ELISA titers (GMT) and fold increase over titers achieved in vaccinated but untreated rabbits. The data show a pronounced enhancement of antibody titer in animals subjected to electroporation.

Table 6

Conditions	Titer	Geometric Mean Titer	Fold Increase
DNA control	36 8 141 86	44	1
6-needle Electro	2660 762 821 289	833	18.93

It will be apparent to those skilled in the art that various modifications may be made in the present invention without departing from the spirit and scope of the present invention. It will be additionally apparent to those skilled in the art that the basic construction of the present invention is intended to cover any variations, uses or adaptations of the invention following, in general, the principle of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains. Therefore, it will

be appreciated that the scope of this invention is to be defined by the claims appended hereto, rather than the specific embodiments which have been presented as examples. All references and documents cited herein are incorporated by reference herein in their entirety.

What Is Claimed Is:

1. A method of enhancing an immune response generated in an animal comprising the steps of:

(A) administering to the animal DNA encoding one or more immunogen of interest; and

(B) applying an electric field to at least the site of such DNA administration.

2. The method of claim 1, wherein said immune response comprises enhanced production of antibody specifically reactive with said immunogen.

3. The method of claim 1, wherein said immune response comprises enhanced production of lymphocytes that produce antibody specifically reactive with said immunogen.

4. The method of claim 1, wherein said animal is a mammal.

5. The method of claim 4, wherein said mammal is selected from the group consisting of a cat, a dog, a horse, a human, a rabbit and a rodent.

6. The method of claim 5, wherein said mammal is a human.

7. The method of claim 1, wherein said immunogen is a protein or peptide of a pathogen.

8. The method of claim 7, wherein said pathogen is selected from the group consisting of a bacterium, a fungus, a yeast, a protozoan, and a virus.

9. The method of claim 8, wherein said pathogen is a bacterium selected from the group consisting of an enteric bacterium, a *Clostridrium*, a *Vibrio*, a *Nocardia, a Corynebacterium*, a *Listeria*, a *Legionella,* a *Bacilli*, a

Staphylococcus, a *Streptococci*, a *Borrelia*, a *Mycobacterium*, a *Neisserium* and a *Trepanoma* bacterium.

10. The method of claim 8, wherein said pathogen is a fungus selected from the group consisting of a *Dermatophyte*, a *Pneumocystis*, a *Trypanosoma*, a *Plasmodium*, a *Candida*, a *Cryptococcus*, a *Histoplasma*, a *Coccidioide*, an *Amoeba* and a Schistosome.

11. The method of claim 8, wherein said pathogen is a virus selected from the group consisting of a parvovirus, an orthomyxovirus, a paramyxovirus, a picornavirus, a papovirus, a herpesvirus, a togavirus, and a retrovirus.

12. The method of claim 11, wherein said pathogen is the retrovirus HIV.

13. The method of claim 12, wherein the DNA administered in step (A) encodes one or more HIV protein or peptide.

14. The method of claim 13, wherein said HIV protein or peptide is the HIV gag protein or a peptide fragment thereof.

15. The method of claim 14, wherein said DNA administered in step (A) comprises a codon-optimized gag-encoding region.

16. The method of claim 13, wherein said HIV protein or peptide is the HIV env protein or a peptide fragment thereof.

17. The method of claim 16, wherein said DNA administered in step (A) comprises a codon-optimized env-encoding region.

18. The method of claim 13, wherein said DNA administered in step (A) encodes both (a) an HIV gag protein or a peptide fragment thereof and (b) an HIV env protein or a peptide fragment thereof.

19. The method of claim 18, wherein said DNA administered in step (A) comprises a codon-optimized gag-encoding region and a codon-optimized env-encoding region

20. The method of claim 1, wherein said DNA encoding one or more immunogen of interest is administered to said animal incorporated in a plasmid form.

21. The method of claim 1, wherein said DNA encoding one or more immunogen of interest is administered to said animal associated with protein or lipid.

22. The method of claim 1, wherein said DNA is administered to said animal by intramuscular or intradermal injection.

23. The method of claim 1, wherein in step (B) said electrical field is applied under electroporation conditions.

24. The method of claim 1, wherein in step (B) said electrical field is applied under iontophoresis conditions.

25. The method of claim 1, wherein said DNA is administered using a device selected from the group consisting of a single needle probe, a bipolar probe and a combination needle and plate probe.

26. An apparatus for enhancing an immune response in an animal comprising:

(A) DNA encoding one or more immunogen of interest;

(B) means for administering said DNA to said animal; and

(C) means for applying an electric field to at least the site of such DNA administration.

27. The apparatus method of claim 26, wherein said immunogen is a protein or peptide of a pathogen.

28. The apparatus of claim 27, wherein said pathogen is selected from the group consisting of a bacterium, a fungus, a yeast, a protozoan, and a virus.

29. The apparatus of claim 28, wherein said pathogen is a bacterium selected from the group consisting of an enteric bacterium, a *Clostridrium*, a *Vibrio*, a *Nocardia, a Corynebacterium*, a *Listeria*, a *Legionella,* a *Bacilli*, a *Staphylococcus*, a *Streptococci*, a *Borrelia*, a *Mycobacterium*, a *Neisserium* and a *Trepanoma* bacterium.

30. The apparatus of claim 28, wherein said pathogen is a fungus selected from the group consisting of a *Dermatophyte,* a *Pneumocystis*, a *Trypanosoma*, a *Plasmodium*, a *Candida,* a *Cryptococcus,* a *Histoplasma,* a *Coccidioide*, an *Amoeba* and a Schistosome.

31. The apparatus of claim 28, wherein said pathogen is a virus selected from the group consisting of a parvovirus, an orthomyxovirus, a paramyxovirus, a picornavirus, a papovirus, a herpesvirus, a togavirus, and a retrovirus.

32. The apparatus of claim 31, wherein said pathogen is the retrovirus HIV.

33. The apparatus of claim 32, wherein the DNA administered in step (A) encodes one or more HIV protein or peptide.

34. The apparatus of claim 33, wherein said HIV protein or peptide is the HIV gag protein or a peptide fragment thereof.

35. The apparatus of claim 34, wherein said DNA administered in step (A) comprises a codon-optimized gag-encoding region.

36. The apparatus of claim 33, wherein said HIV protein or peptide is the HIV env protein or a peptide fragment thereof.

37. The apparatus of claim 36, wherein said DNA administered in step (A) comprises a codon-optimized env-encoding region.

38. encodes both (a) an HIV gag protein or a peptide fragment thereof and (b) an HIV env protein or a peptide fragment thereof.

39. The apparatus of claim 38, wherein said DNA administered in step (A) comprises a codon-optimized gag-encoding region and a codon-optimized env-encoding region.

40. The apparatus of claim 26, wherein said DNA encoding said one or more immunogens of interest is incorporated in a plasmid form.

41. The apparatus of claim 26, wherein said DNA encoding one or more immunogen of interest is associated with protein or lipid.

42. The apparatus of claim 26, wherein said means for administering said DNA to said animal accomplishes intramuscular or intradermal administration of said DNA.

43. The apparatus of claim 26, wherein said electrical field is produced under electroporation conditions.

44. The apparatus of claim 26, wherein said electrical field is produced under iontophoresis conditions.

45. The apparatus of claim 26, wherein said means for administering said DNA is a device selected from the group consisting of a single needle probe, a bipolar probe and a combination needle and plate probe.

FIGURE 1A

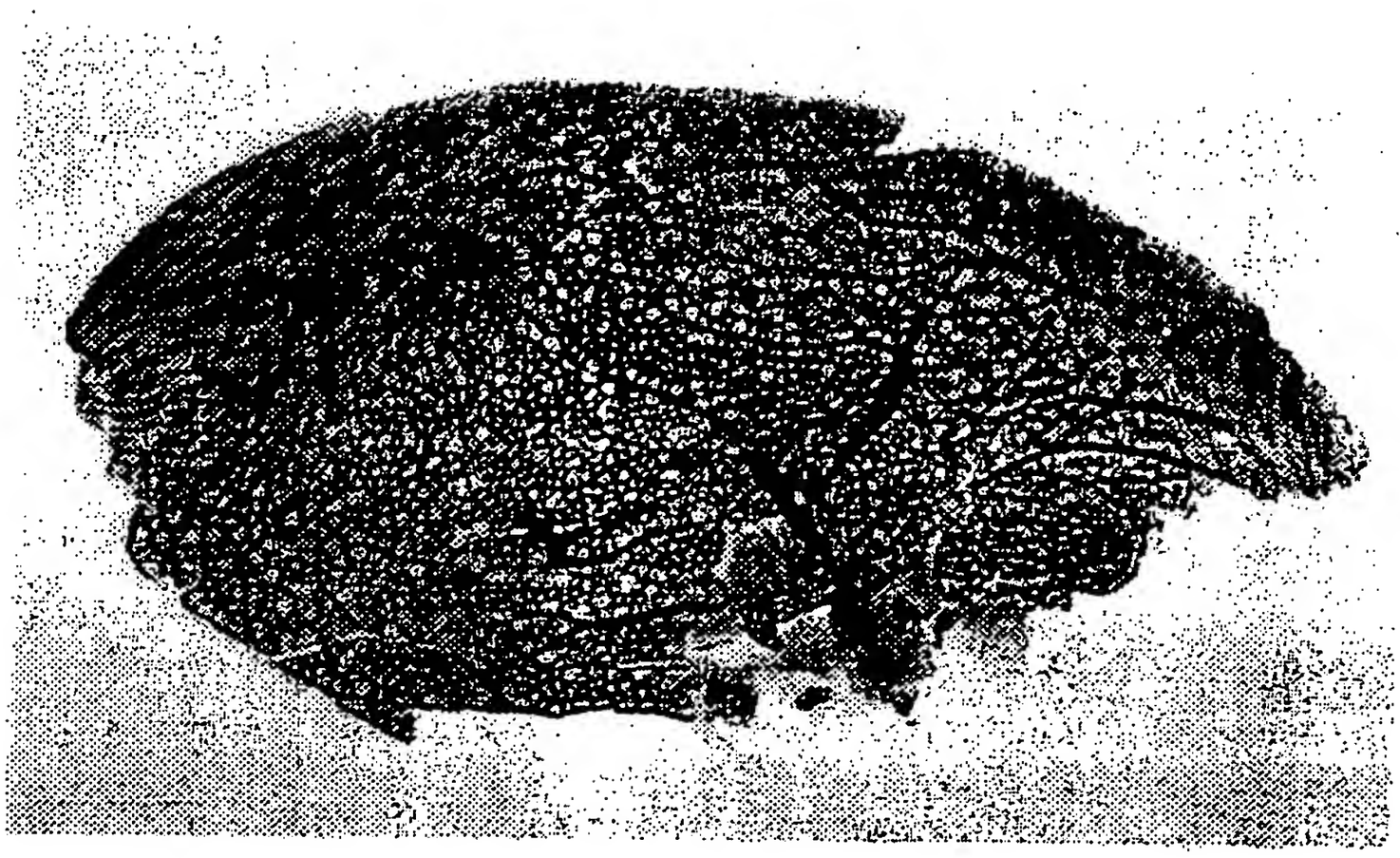


FIGURE 1B

FIGURE 2

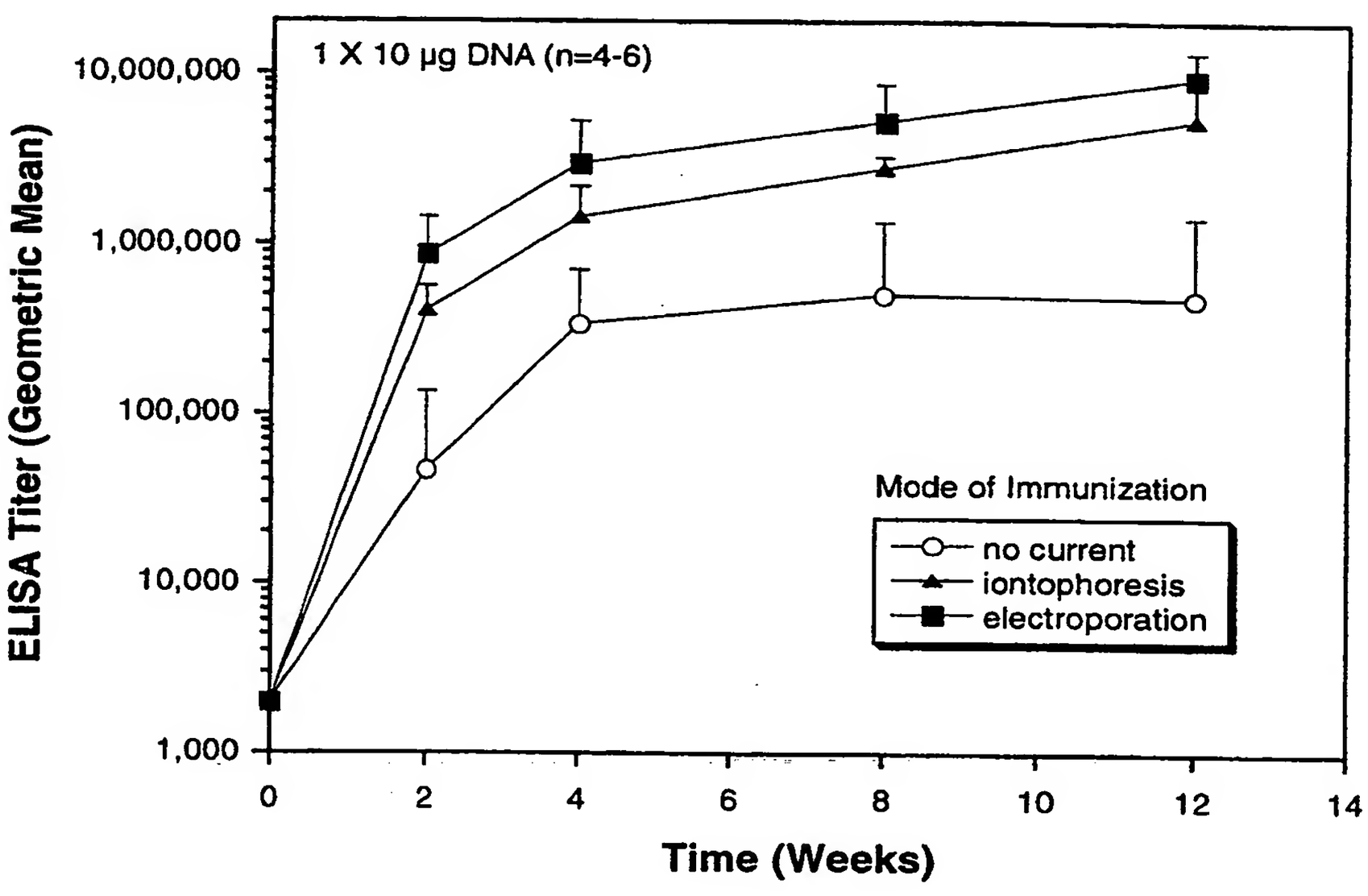

1 X 10 µg DNA (n=4-6)
ELISA Titer (Geometric Mean)
10,000,000
1,000,000
100,000
10,000
1,000
0
2
4
6
8
10
12
14
Time (Weeks)
Mode of Immunization
no current
iontophoresis
electroporation

FIGURE 3

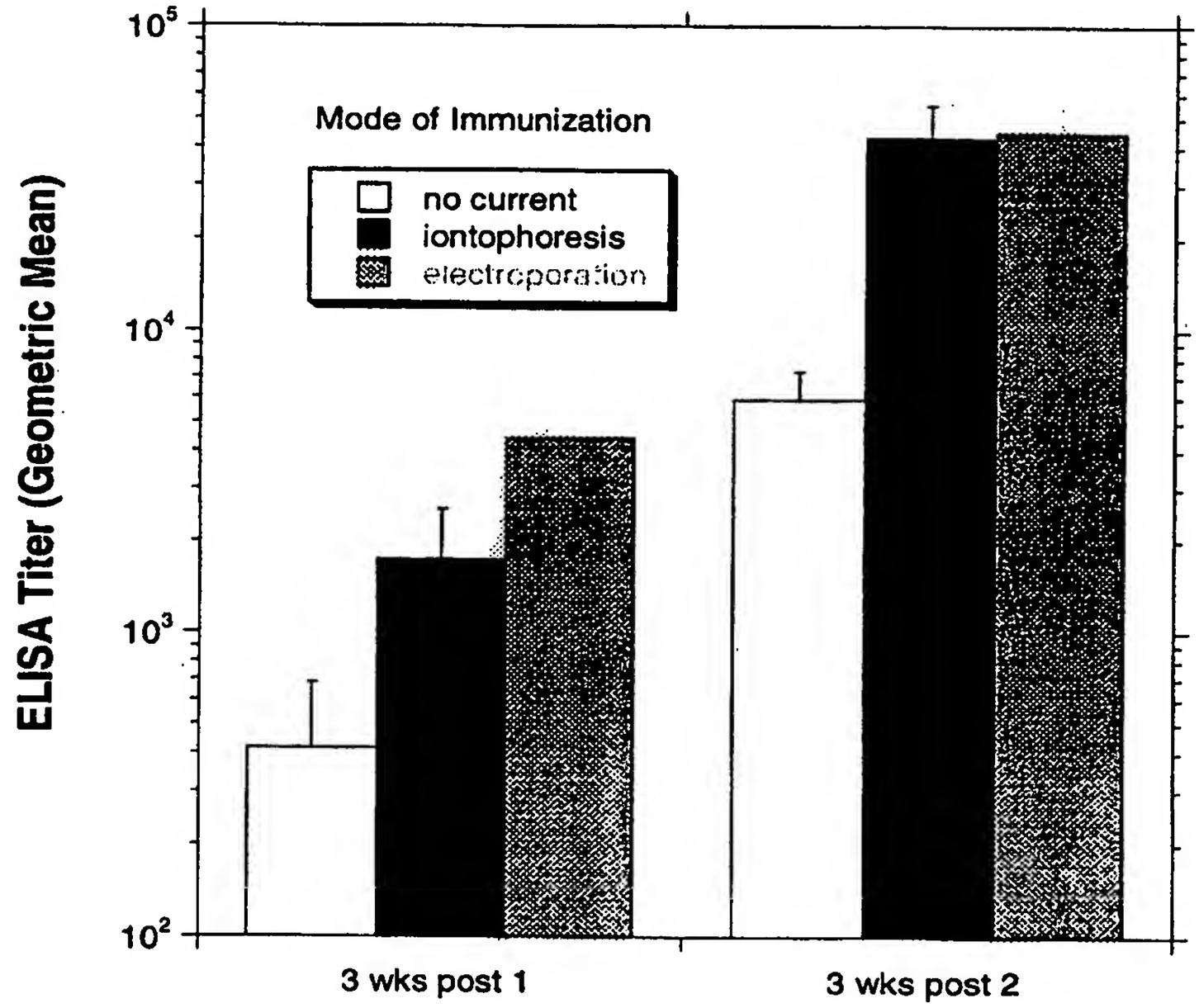
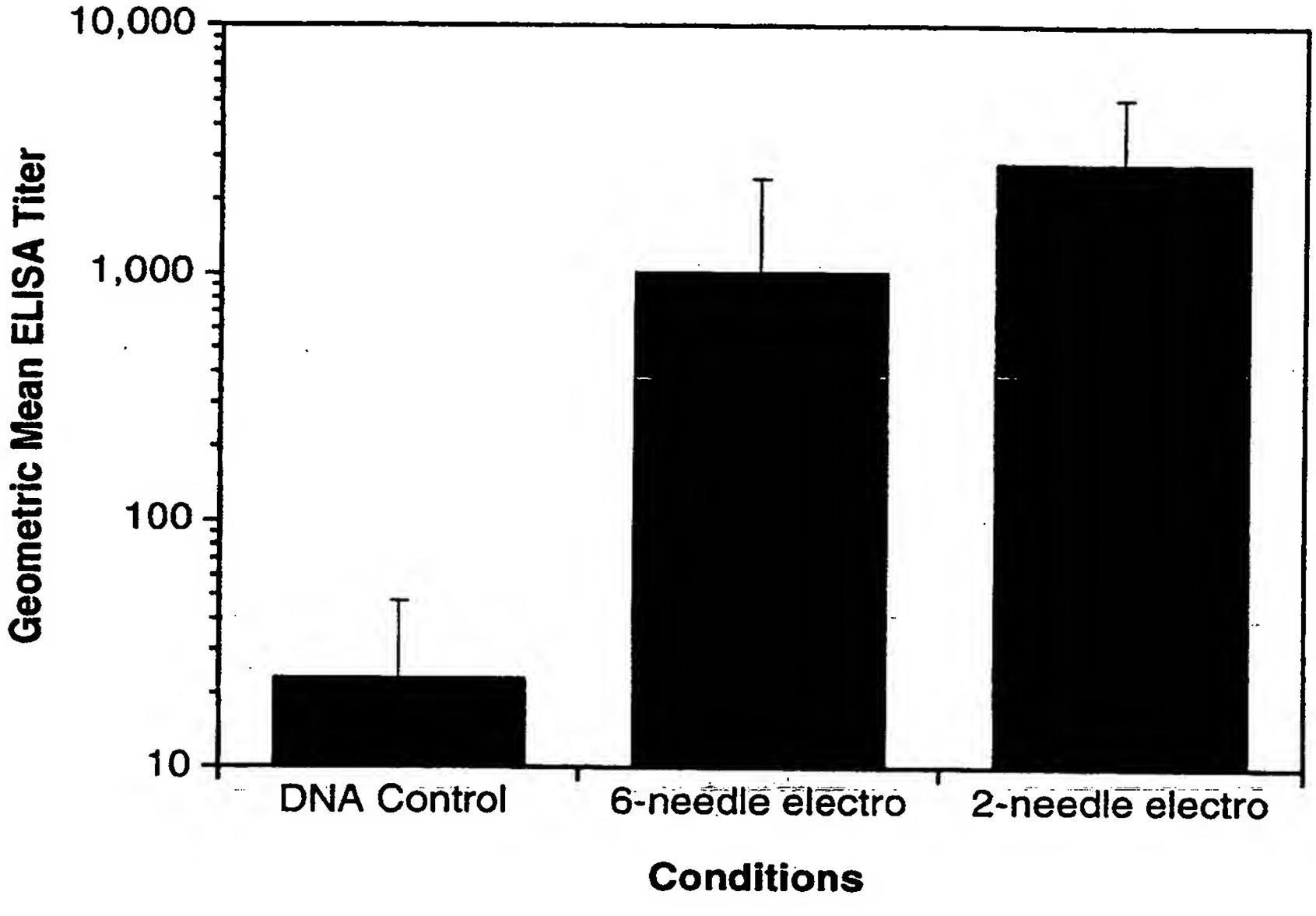


FIGURE 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/02831

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/70, 45/00; A61N 1/30, 1/00; C12M 1/42
US CL : 514/44; 424/278.1; 604/20; 607/116; 435/285.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/278.1; 604/20; 607/116; 435/285.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE BIOSIS EMBASE CAPLUS BIOTECHDS WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	NOMURA et al. In vivo induction of cytotoxic T lymphocytes specific for a single epitope introduced into an unrelated molecule. Journal of Immunological Methods. 1993, Vol. 193, pages 41-49, see entire document, especially, abstract; Fig. 1, page 42; page 44, column 1, second full paragraph; and Fig. 4, page 46.	1, 4, 5, 7, 8, 11, 20, 22, 23, 26-29, 31, 42, 43 ---------- 2, 3, 6, 9, 10, 12-19, 21, 24, 25, 30, 32-41, 44, 45

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
13 APRIL 2000	21 JUN 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RICHARD SCHNIZER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/02831

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,830,877 A (CARSON et al.) 03 November 1998, entire document, especially col. 11, lines 3-15; col. 20, lines 24-32; paragraph bridging columns 20 and 21; col. 24, lines 10-18; col. 37, lines 50 and 51; and Fig. 22.	2, 3, 24, 25, 44, and 45
Y	US 5,593,972 A (WEINER et al.) 14 January 1997, see entire document, especially abstract; col. 6, lines 42-49; col. 13, line 5-19; col. 23, lines 10-20; and col. 34, lines 15-26.	5-19, and 27-39
Y	US 5,786,464 A (SEED) 28 July 1998, see entire document, especially col. 1, lines 10-12 and 29-34; col. 2, lines 7-13 and 51-55; and column 41, claims 12-14.	8, 11-19, 27, 31-39
Y	EP 0 438 078 A2 (ROSSI et al.) 24 July 1991, see entire document, especially abstract.	25 and 45

ISDOCID: <WO 0045823A1 I >

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 August 2000 (10.08.2000)

PCT

(10) International Publication Number
WO 00/45823 A1

(51) International Patent Classification[7]: A61K 31/70, 45/00, A61N 1/30, 1/00, C12M 1/42

(21) International Application Number: PCT/US00/02831

(22) International Filing Date: 7 February 2000 (07.02.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/118,996 8 February 1999 (08.02.1999) US
60/129,189 14 April 1999 (14.04.1999) US

(71) Applicants: **CHIRON CORPORATION** [US/US]; 4560 Horton Street, Emeryville, CA 94608-2916 (US). **EMED CORPORATION** [US/US]; 651 Campus Drive, St. Paul, MN 55112 (US).

(72) Inventors: **SELBY, Mark**; 136 Galewood Circle, San Francisco, CA 94131 (US). **GOLDBECK, Cheryl**; 725 Camino Ricardo, Moraga, CA 94556 (US). **PERTILE, Terry**; 3627 Rodeo Drive, Blaine, MN 55449 (US). **WALSH, Robert**; 17185 Jackson Trail, Lakeville, MN 55144 (US). **LIU, Margaret, A.**; 3656 Happy Valley Road, Lafayette, CA 94549 (US). **ULMER, Jeffery**; 160 Summerfield Street, Danville, CA 94506 (US).

(74) Agent: **AUERBACH, Jeffery, I.**; Liniak, Berenato, Longacre & White, LLC, 6550 Rock Spring Drive, Suite 240, Bethesda, MD 20817 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(48) Date of publication of this corrected version:
11 October 2001

(15) Information about Correction:
see PCT Gazette No. 41/2001 of 11 October 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/45823 A1

(54) Title: ELECTRICALLY-MEDIATED ENHANCEMENT OF DNA VACCINE IMMUNITY AND EFFICACY IN VIVO

(57) Abstract: Electrically-mediated delivery technology has been applied to DNA vaccines and substantially higher immune responses have been achieved. In mice and rabbits vaccinated with DNA encoding HIV genes, when administered with constant electric current or constant electric voltage, up to twenty-fold higher immune responses were achieved compared with application of DNA vaccines alone. The increase was achieved under conditions of both constant current (iontophoresis) and constant voltage (electroporation).

TITLE OF THE INVENTION

ELECTRICALLY-MEDIATED ENHANCEMENT OF DNA VACCINE IMMUNITY AND EFFICACY IN VIVO

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Provisional Patent Applications Serial Numbers 60/118996 (filed February 7, 1999) and 60/129189 (filed April 14, 1999), both of which applications are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates generally to the use of electrical pulses to enhance DNA vaccine efficacy *in vivo*. More particularly, the present invention relates to the use of electrical pulses to enhance HIV DNA vaccine efficacy *in vivo* and even more particularly to the use of electrical pulses to enhance HIV gag DNA vaccine efficacy *in vivo*.

BACKGROUND OF THE INVENTION

Vaccines composed of live, attenuated pathogens have long been used to provide and/or enhance immunity. Recently, however, the ability to introduce DNA into cells and tissues has led to the proposal that DNA vaccines could be used in lieu of pathogens to provide immunity (for review, see Donnelly *et al.* (1997) *Ann. Rev. Immunol. 15*:617-648).

Although DNA vaccines offer the potential for greater safety, efficacy and protection than that provided by conventional vaccines, the delivery of such DNA to cells has presented several problems (Lai and Bennett (1998) *Crit. Rev. Immunol. 18*:449-484). DNA vaccines given orally have been reported to be

incapable of eliciting an immune response (see Manikan *et al.* (1997) *Crit. Rev. Immunol. 17*:139-154). Likewise, introduction of DNA into the dermis has been found to be complicated both by the susceptibility of the basal cells of the epidermis to transformation, and by rapid turnover of epidermal cells that leads to the expulsion of much of the administered DNA (Lai and Bennett (1998) *Crit. Rev. Immunol. 18*:449-484). Introduction of DNA into muscle cells has been effective to confer immunity in some cases, however, it has been reported that muscle cells do not seem capable of expressing molecules required for efficient antigen presentation (Goebels *et al.* (1992) *J. Immunol. 149*:661-667; Hohfield and Engel (1994) *Immunol. Today 15*:269-274; Michaelis *et al.* (1993) Amer. J. Pathol. 143:1142-1149). Accordingly, a need exists to enhance DNA vaccine efficacy.

The use of electric current has facilitated gene delivery *in vitro* and *in vivo*. Transient discontinuities in the plasma membranes of cells can be induced by short pulses of high-voltage electric current. These discontinuities allow substances, such as DNA to passively enter cells directly into the cytoplasm, thereby avoiding the indirect and inefficient route of endocytosis. As a consequence, more DNA is delivered inside cells and a greater degree of transfection occurs. This process, termed electroporation is widely used for facilitation of transfection of cells *in vitro*.

Recently, the use of electric current to mediate transfer of genes *in vivo* has been reported. Successful transfer of genes has been accomplished for cells of the skin (Titomirov *et al.* (1991) *Biochim. Biophys. Acta 1088*: 131-134; Nomura *et al.* (1996) *J. Immunol. Meth. 193*: 41-49), liver (Heller *et al.* (1996) *FEBS Lett. 389*:225-228; Suzuki *et al.* (1998) *FEBS Lett. 425*: 436-440), tumors (Nishi *et al.* (1996) *Cancer Res. 56*: 1050-1055; Nishi *et al.* (1997) *Hum. Cell 10*: 81-86; Rols *et al.* (1998) *Nature Biotechnol. 16*: 168-171), oviduct (Ochiai *et al.* (1998) *Poult Sci. 77*:299-302), and muscle (Aihara and Miyazaki (1998) *Nature Biotechnol*: *16*: 867-870). In most cases, protein expression was demonstrated, and in some cases biological effects were noted, such as regression of tumors or increased hematocrit after inoculation of erythropoietin DNA (*Rizutto et al.* (1999) *Proc. Natl. Acad.*

Sci. (USA) 96:6417-6422). In one case, induction of an immune response was detected in mice after electroporation *in vivo* with DNA encoding a fusion protein containing a CTL epitope from influenza nucleoprotein (Nomura *et al.* (1996) *J. Immunol. Meth. 193*: 41-49).

A technology related to electroporation, termed iontophoresis, involves the application of an electric field to facilitate movement of charged molecules, such as "naked DNA," in tissue and across biological membranes. Iontophoresis, which involves lower electric current than what is required for electroporation, has been widely used for transdermal delivery of drugs and oligonucleotides.

The efficacy of DNA vaccines in preclinical models has been well documented (for review see Donnelly *et al.* (1997) *Ann. Rev. Immunol. 15*:617-648). The magnitude of immune responses, however, induced in primates is generally lower than that in small animals, and the amount of DNA required for effective immunization of primates is much higher (mg versus μg) (for example, see Kent *et al.* (1998) *J. Virol. 72*:10180-10188; Gramzinski *et al.* (1998) *Molec. Med. 4*:109:118; Richmond *et al.* (1998) *J. Virol. 72*: 9092-9100). In addition, several phase I human clinical studies have been conducted with little or no immune responses reported (Calarota *et al.* (1998) *Lancet 351*: 1320-1325; MacGregor *et al.* (1998) *J. Infect. Dis. 178*:92-100; McClements-Mann *et al.* (1997) Amer. Soc. Virol. Ann. Meet. Abstr. (Vancouver, Canada), p. 115).). In one case, however, cytotoxic T lymphocytes were induced in human volunteers by a malaria DNA vaccine, but no antibodies were detected (Wang *et al.* (1998) *Science 282*:476-480. Therefore, the potency of DNA vaccines must be increased to enable this technology for successful human application. The present invention demonstrates the enhancement of DNA vaccine potency in animals using electrically-mediated delivery of DNA.

SUMMARY OF THE INVENTION

It is a primary object of the invention to provide electrically-mediated enhancement of DNA vaccine efficacy *in vivo*. This object is achieved through the use of electrical current to facilitate gene delivery to cells and tissue. In accordance with an embodiment of the invention, DNA encoding the immunogen of interest is administered parenterally followed by the application of electrical current in either the iontophoresis or electroporation range.

It is a further object of the invention to provide electrically-mediated enhancement of HIV DNA vaccine efficacy *in vivo*. Preferably, the HIV DNA is HIV gag DNA. In embodiments of the invention, such DNA is incorporated into a plasmid and is injected either via an intramuscular (i.m.) or intradermal (i.d.) route.

In detail, the invention provides, a method of enhancing an immune response generated in an animal comprising the steps of:

(A) administering to the animal DNA encoding one or more immunogen of interest; and

(B) applying an electric field to at least the site of such DNA administration.

The invention particularly concerns the embodiment of the above method in which the immunogen is a protein or peptide of a pathogen (especially a bacterium, a fungus, a yeast, a protozoan, or a virus). The invention is particularly concerned with the embodiment of the above method wherein the pathogen is the retrovirus HIV, and wherein the DNA administered in step (A) encodes one or more HIV protein or peptide (especially the HIV gag and/or env proteins or a peptide fragment of either, and most preferably codon-optimized DNA molecules encoding these immunogens).

The invention particularly concerns the embodiment of the above method in which the electrical field is applied under electroporation conditions or under iontophoresis conditions.

The invention additionally provides an apparatus for enhancing an immune response in an animal comprising:

(A) DNA encoding one or more immunogen of interest;

(B) means for administering the DNA to the animal; and

(C) means for applying an electric field to at least the site of such DNA administration.

The invention particularly concerns the embodiment of the above apparatus in which the immunogen is a protein or peptide of a pathogen (especially a bacterium, a fungus, a yeast, a protozoan, or a virus). The invention is particularly concerned with the embodiment of the above apparatus wherein the pathogen is the retrovirus HIV, and wherein the administered DNA encodes one or more HIV protein or peptide (especially the HIV gag and/or env proteins or a peptide fragment of either and most preferably codon-optimized DNA molecules encoding these immunogens).

The invention additionally concerns the embodiment of the above apparatus in which the means for administering the DNA to the animal accomplishes the intramuscular or intradermal administration of the DNA.

The invention additionally concerns the embodiment of the above apparatus in which the electrical field is produced under electroporation or iontophoresis conditions.

The invention additionally concerns the embodiment of the above apparatus in which the means for administering the DNA is a device selected from the group consisting of a single needle probe, a bipolar probe and a combination needle and plate probe.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and **Figure 1B** show the expression of β-galactosidase in mouse muscles that had received β-galactosidase-encoding DNA either without additional treatment (**Figure 1A**) or after electroporation (**Figure 1B**).

Figure 2 shows the ability of electroporation and iontophoresis to enhance the antibody responses of mammals after a single inoculation with DNA encoding the HIV gag protein.

Figure 3 shows the effect of vaccine boosting on antibody responses in mammals inoculated with DNA encoding the HIV gag protein. Note the enhanced immune responses induced by electroporation and iontophoresis even after the booster immunization.

Figure 4 shows the efficacy of electroporation on the anti-HIV gag antibody response of mammals inoculated with a DNA vaccine encoding HIV gag, followed by immunization with recombinant gag protein. Note the enhanced levels of booster response in rabbits that had been primed with DNA and electroporation compared to animals primed with DNA alone.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the enhancement of DNA vaccine efficacy by electrically-mediated administration of the DNA *in vivo*. The recipient of the DNA vaccine may be any mammal (especially a cat, a dog, a horse, a human, a rabbit or a rodent). The invention particularly contemplates that the recipient of the DNA vaccine may be a human.

The DNA vaccine that is administered in accordance with the present invention encodes one or more immunogens. As used herein, an immunogen is a protein or a peptide (i.e., a fragment of a protein) that contains at least one epitope such that the immunogen induces an enhanced immune response in a recipient mammal. As used herein, a treatment or procedure is said to enhance an immune

response if the treatment or procedure increases the extent, duration or degree of the response beyond that observed in the absence of such treatment or procedure. The enhanced immune responses of the present invention include the enhanced production of antibody that is specifically reactive with the immunogen, and the enhanced production of lymphocytes that produce such antibody. An antibody is said to be specifically reactive with an immunogen if it binds to the immunogen in an immunologically relevant manner.

Any of a variety of DNA vaccines may be used in accordance with the present invention include those (for review, see Donnelly *et al.* (1997) *Ann. Rev. Immunol. 15*:617-648; Manikan *et al.* (1997) *Crit. Rev. Immunol. 17*:139-154; Alarcon *et al.* (1999) Adv. Parasitol. 42: 343-410; Lai and Bennett (1998) *Crit. Rev. Immunol. 18*:449-484; Tuteja (1999) *Crit. Rev. Biochem. Molec. Biol. 34*:1-24). In a preferred embodiment, the DNA vaccine of the present invention will encode more than one epitope. Thus, for example, the administered DNA may encode all of the epitopes of a protein associated with HIV (such as the gag or env protein). Alternatively, the administered DNA may encode only a peptide of such protein that contains one (or fewer than all) of the protein's epitopes.

The present invention contemplates that the immunogens encoded by the DNA vaccine of the present invention may comprise a protein or peptide of a pathogen. Such pathogen may be any of a wide group of bacteria (e.g., *E. coli* strains and strains of other enterics (e.g., *Salmonella*), *Clostridria*, *Vibrio*, *Corynebacteria*, *Listeria*, *Nocardia*, *Legionella*, *Bacilli* (especially *B. anthracis*), *Staphylococcus*, *Streptococci* (especially beta-hemolytic *Streptococci* and *S. pneumoniae*), *Borrelia*, *Mycobacterium* (especially *M. tuberculosi*), *Neisseria* (especially *N. gonorrhoeae*), *Trepanoma*, etc.), viruses (e.g., parvoviruses, orthomyxoviruses (especially those causing influenza), paramyxoviruses, picornaviruses (especially rhinoviruses or polioviruses), papoviruses, herpesviruses, togaviruses, retroviruses (especially HIV), rhabdoviruses, etc.), and lower eukaryotes (e.g., fungi, protozoa, yeast, helminths, nematodes, etc. (especially *Dermatophytes*, *Pneumocystis*, *Trypanosoma*, *Plasmodium*, *Candida*,

Cryptococcus, *Histoplasma*, *Coccidioides*, amoeba, schistosomes, etc.). Alternatively, the immunogens of the present invention may encode antigens that are produced by aberrant or diseased cells of the recipient (e.g., cancer cells, etc.), such that the recipient animal will form antibodies that will attack such cells.

The immunogens encoded by the DNA vaccine of the present invention may be related to one another, may be clinically related, or may be unrelated to one another. As used herein, immunogens are related to one another if the immune responses that they induce elicit antibodies that bind to the same cell, microbe, virus, etc. For example, DNA that encodes epitopes of the gag or env protein would encode related immunogens. Immunogens are said to be clinically related to one another if the immune responses that they induce elicit antibodies that bind to different cells, microbes, viruses, etc. that are associated with the same clinical condition. For example, individuals suffering from Acquired Immunodeficiency Syndrome (AIDS) may develop infections caused by the bacterium *Listeria monocytogenes*, and by the yeast *Candida*. DNA that encodes epitopes of a *Listeria monocytogenes* protein and a *Candida* protein would encode clinically related immunogens. Alternatively, the DNA vaccine of the present invention may encode an epitope of a poliovirus and an epitope of a measles virus, and thus provide unrelated immunogens.

Most preferably, the DNA of the DNA vaccine of the present invention will contain regulatory elements (promoters, translation initiation sites, etc.) operably linked to the immunogen-encoding sequences and sufficient to permit the protein expression of the immunogen. Alternatively, the administered DNA will not contain such regulatory elements, and will require cellular processes (such as recombination or integration into nuclear or mitochondrial DNA, etc.) in order to produce the encoded immunogen.

The DNA vaccine of the present invention may comprise more than one molecular species of DNA. Such multiples species may contain the same DNA sequence (e.g., a mixture of circular and linearized plasmids), or may contain

different DNA sequences encoding the same immunogen (e.g., a mixture of DNA molecules of different length all of which contain a particular immunogen-encoding sequence), or may contain DNA sequences encoding different immunogens. The administered DNA can be either "naked" DNA (i.e., free of associated protein or lipids), or may be complexed with protein or lipids or other molecules. For example, the DNA can be administered with a local anesthetic such as bupivicaine or a myotoxin such as cardiotoxin, or with proteins that assist in the efficient presentation of antigen (e.g., CD80, CD86, etc.) (Tuteja (1999) *Crit. Rev. Biochem. Molec. Biol. 34*:1-24). The DNA may encode only the desired immunogen or immunogens, or may encode other additional proteins or peptides that may be linked or unlinked to the immunogen and that enhance immunogen stability or immunogenicity. The DNA may also encode protein extraneous to the immunogenicity of the immunogen that is encoded by the DNA; such extraneous protein may likewise be linked or unlinked to the immunogen. The DNA of the DNA vaccine of the present invention may contain untranslated or untranscribed DNA.

The DNA can be incorporated into a recombinant expression vector such as a chimeric virus, a plasmid DNA, etc. The DNA is preferably dissolved or suspended in a buffer or other solution (e.g., 5% dextrose).

In a particularly preferred embodiment, DNA, preferably in the form of plasmid DNA, is administered (especially by injection) into tissue and voltage pulses are applied between electrodes disposed in the tissue, thus applying electric fields to cells of the tissue. The electrically-mediated enhancement covers administration using either iontophoresis or electroporation *in vivo*. Suitable techniques of electroporation and iontophoresis are provided by Singh *et al.* (1989) *Drug Des. Deliv. 4*:1-12; Theiss U *et al.* (1991) *Methods Find. Exp. Clin. Pharmacol. 13*:353-359; Singh and Maibach (1993) *Dermatology. 187*:235-238; Singh and Maibach (1994) *Crit. Rev. Ther. Drug Carrier Syst. 11*:161-213; Su *et al.* (1994) *J. Pharm. Sci. 83*:12-17; Costello *et al.* (1995) *Phys. Ther. 75*:554-563; Howard *et al.* (1995) *Arch. Phys. Med. Rehabil. 76*:463-466; Kassan *et al.* (1996)

J. Amer. Acad. Dermatol. 34:657-666; Riviere *et al.* (1997) *Pharm. Res. 14*:687-697; Zempsky *et al.* (1998) *Amer. J. Anesthesiol. 25*:158-162; Muramatsu *et al.* (1998) *Int. J. Mol. Med. 1*:55-62; Garrison J. (1998) *Med. Device Technol. 9*:32-36; Banga *et al.* (1998) *Trends Biotechnol. 16*:408-412; Banga *et al.* (1999) *Int. J. Pharm. 179*:1-19; Singh *et al.* (1999) *Anticancer Drugs. 10*:139-146; Neumann *et al.* (1999) *Bioelectrochem. Bioenerg. 48*:3-16; and Heiser (2000) *Methods Mol. Biol. 130:*117-134. Whereas any suitable route of inoculation may be employed, of intra-muscular (i.m.), intra-dermal (i.d.), or sub-cutaneous (s.c.), i.m. injection is the most efficacious. Enhanced immune responses are, however, also seen after i.d. injections.

The nature of the electric field generated in accordance with the present invention is determined by the nature of the tissue, the size of the selected tissue and its location. It is desirable that the field be as homogeneous as possible and of the correct amplitude. The use of insufficient or excessive field strength is to be avoided. As used herein, a field strength is excessive if it results in the lysing of cells. A field strength is insufficient if it results in a reduction of efficacy of 90% relative to the maximum efficacy obtainable. The electrodes may be mounted and manipulated in many ways known in the art.

The waveform of the electrical signal provided by the pulse generator can be an exponentially decaying pulse, a square pulse, a unipolar oscillating pulse train or a bipolar oscillating pulse train. The waveform, electric field strength and pulse duration are dependent upon the type of cells and the DNA that are to enter the cells via electrical-mediated delivery and thus are determined by those skilled in the art in consideration of these criteria.

Any number of known devices may be used for delivering the DNA vaccine and generating the desired electric field. Examples of suitable devices include, but are not limited to, a single needle probe, a bipolar probe and a combination needle and plate probe. The single needle probe exemplified herein is a single stainless steel needle, with an insulation stop that provides preferably about 3mm of active

zone. The single needle serves as the negative electrode and the plasmid delivery device. The positive electrode is a hypodermic needle located in the opposite leg or arm of the recipient patient or test animal. The bipolar probe exemplified herein contains two stainless steel needles preferably about 3mm in length and separated by a distance of preferably about 0.4cm. One needle carries a positive charge and one needle carries a negative charge. The combination needle and plate probe exemplified herein contains two stainless steel needles preferably about 3 mm in length and separated by a distance of preferably about 0.4cm. The needles are insulated except for the distal 1mm. Both needles serve as the negative electrodes. The needles protrude from a stainless steel block. The block sits on the surface of the skin and serves as the positive electrode. The separation distance between the nearest active area on the block to the nearest active area on the needles is preferably about 2.5mm. The needles are insulated from direct contact with the stainless steel block.

Preferred electrical field conditions for i.m. administration are as follows: 50mA for 10msec for 5 pulses then rotated 90° (i.e., orthogonally) for 5 additional pulses; 120V for 10 msec for 5 pulses then rotate orthogonally for 5 additional pulses when using the bipolar probe; and 80V for 10msec for 5 pulsed then rotate orthogonally for 5 additional pulses when using the combination plate and needle probe. Preferred electrical field conditions for i.d. administration are as follows: 50mA for 50msec for 5 pulses then rotate orthogonally for 5 additional pulses; and 120 V for 50 msec for 5 pulses then rotate orthogonally for 5 additional pulses when using the bipolar probe.

Preparations of DNA for parenteral administration include but are not limited to sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate.

The increased DNA vaccine potency observed after iontophoresis or electroporation may reflect a facilitation, by the electric current, of the distribution

of DNA within the injected tissue and/or uptake of DNA by cells, leading to increased transfection. The ensuing increase in the amount of antigen expressed by cells is likely to have played a role in the elevated immune responses. Alternatively, or in addition, infiltration of inflammatory cells (in response to the electric current) could have an "adjuvant" effect on the produced antigen. The present invention demonstrates that DNA vaccine potency can be increased by application of electric current. The results indicate that a significant limitation to efficient transfection of cells *in vivo* by naked DNA vaccines in the past (possibly accounting for the lack of efficacy of DNA vaccines in larger animals, such as primates, in the past) has been the distribution of the introduced DNA within tissue and/or uptake of DNA by cells. Iontophoresis and electroporation (as well as equivalent means for facilitating the delivery of DNA into cells and tissue can be used to surmount this problem and enable the development of DNA vaccines.

Having now fully described the invention, the same will be further illustrated by way of the following examples, which are meant solely to illustrate the invention and are not to be construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention.

Example 1
Materials And Methods For *In Vivo* Electrical-Enhanced Delivery Of DNA.

Bacterial Strain and plasmid preparation

The bacteria Escherichia coli strain HB101 were transformed with the plasmids pCMV HIV gag prepared as described in U.S. Provisional Patent Application 60/114495, filed 31 December 1998, or pCMV KM LUC encoding firefly luciferase reporter gene (LUC). In brief, a luciferase expression plasmid was obtained from Promega Corporation (Madison, WI). *E. coli* strain XL-1 Blue (Stratagene, La Jolla), carrying the expression plasmid, was grown in LB; antibiotic selection employed 50 μg/ml of ampicillin. Plasmids were purified using Qiagen Endo Free Plasmid Maxi Kits (Qiagen, Inc., Chatsworth, CA) according to the

manufacturer instructions and resuspended in 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL).

The plasmid pCMV HIV gag was used as a source of gag-encoding DNA. The plasmid expresses high levels of HIV-1 gag, due to a potent CMV promoter with intron A and a codon-optimized gag encoding region (see U.S. Provisional Patent Application Serial No. 60/168,471, filed December 1, 1999). The plasmid was grown in *E. coli* strain HB101, purified using a Qiagen Endofree Plasmid Giga kit, (Qiagen, Inc.) and resuspended in 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL). Plasmid concentrations were analyzed by measuring absorbance at 260 nm.

Expression of the encoded antigens was verified by transient expression studies in B16 cells. One µg of each plasmid DNA was used for Lipofectin (Gibco/BRL) transfection following the manufacturers protocol; $5x10^5$ cells were used per 3 cm tissue culture dish; incubation time for DNA/Lipofectin on cells was for 4 hours. Supernatants were harvested 36 hours after removal of the DNA/Lipofectin solution and cells were lysed in 500 µl phosphate buffered saline (PBS)/0.5% TritonX100 (Mallinckrodt). Luciferase activity in cell lysates was detected by commercial Luciferase Reporter Gene Assay (Roche, Indianapolis, IN).

Immunization Procedure:

Female 6-8 week old CB6F1 or BalbC mice (Charles River) were anesthetized using 4 parts ketamine HCl, 100mg/ml stock solution, (Fort Dodge Animal Health, Fort Dodge, Iowa) 1 part xylazine, 20mg/ml, (LLoyd Labs, Shenandoah, Iowa). The mice received 1µl per gm of body weight intramuscularly in the posterior thigh. The anterior tibialis (TA) muscle was shaved and the animals were injected with 10 µg of plasmid in a volume of 50 µl. To control needle depth, the syringe was covered with polyethylene tubing (i.d. 0.38) to expose only the bevel. The animals were injected intramuscularly, intradermally or subcutaneously. For each of the types of injections, an electrical field was then applied to the animals except to the control group of animals. One group of

animals received an electrical field in the iontophoresis range. That is, using a single needle probe set-up 50 mA at a 10 msec pulse width, 1 Hz frequency for a total of 60 pulses were delivered. Another group of animals received an electrical field in the low electroporation range. That is, 40 V at 10 msec pulse width, 1 Hz frequency for 5 pulses were delivered plus 5 additional pulses were delivered after the probe was turned in an orthogonal direction to the first set of 5 pulses. Another group of animals received an electrical field in the high electroporation range. That is 80 V, at a 10 msec pulse width, 1 Hz frequency for 5 pulses were delivered plus 5 additional pulses were delivered after the probe was turned in an orthogonal directed to the first set of 5 pulses. Serum samples were collected at 2, 4, 8 and 12 week intervals and analyzed by the below-outlined procedures. The results of this experiment showed enhanced antibody titers in the animals inoculated by the i.m. route with enhancement ranging from 8- to 20-fold.

Immunoassays:

The mouse anti-p55 IgG antibodies were measured by one of two methods, chemi-luminescent or colormetric ELISA assays.

Chemi-luminescent ELISA

MicroLite 2, 96 well flat bottom plates (Dynes Technologies, Chantilly, VA) were coated with HIV p24 protein at 5µg/ml in 10mM tris pH=7.5, 50 µl per well and incubated at 4°C overnight. The plates were washed 3X with wash buffer [1X AquaLite® Wash Buffer (SeaLite Sciences, Inc. Bogart, GA) containing 0.3% Tween 20 (Sigma, St. Louis, MO)], and blocked at 37°C for 1 hour with 150 µl/well blocking buffer [1X Streptavidin AquaLite® Assay buffer (SeaLite Sciences, Inc. Bogart, GA) containing 5% goal serum]. The plates were washed 3X and the test sera were diluted 1/300 or 1/9000 followed by serial 3-fold dilutions in the blocking buffer. A volume of 50 µl of each dilution was added per well and the plates were incubated at 37°C for 1 hour. The plates were washed 6X and incubated for 1 hour at 37°C with 50 µl/well of Goat anti-mouse IgG –Biotin (Sigma St. Louis, MO), diluted 1/1000 in block buffer. After washing 6X, the plates were incubated at 37°C for 1 hour with Streptavidin-Aqualite® (SeaLite

Sciences, Inc. Bogart, GA), diluted 1/500 in wash buffer, 50 µl/well. The plates were washed 6X and stored in wash buffer until reactivity was measured on the luminometer (MLX, Dynex Technologies, Chantilly, VA). Setting for the luminometer – mode: Integrate Flash, Gain: High, Data: Table, Delay window: 0.00 sec., Integrate window: 3.00 sec., Before peak: 0.10 sec., After peak: 2.00 sec, calibrate on each well. The plates were tapped dry and put into the luminometer. Fifty microliters of 1X AquaLite® Trigger Buffer (SeaLite Sciences, Inc. Bogart, GA) were automatically dispensed per well and the relative light units (RLU) measured. Endpoint titers were calculated as the inverse of the dilution that yields an RLU equal to the background plus 5 times the standard deviation.

Colormetric ELISA

Wells of Immulon 2 HB "U" bottom microtiter plates (Dynex Technologies, Chantilly, VA) were coated with HIV p55 protein at 5 µl/ml in PBS, 50 µl per well, and incubated at 4°C overnight. The plates were washed 6X with wash buffer [PBS, 0.1% tween (Sigma, St. Louis, MO)] and blocked at 37°C for 1 hour with 150µl/well blocking buffer [PBS, 0.1% tween 20 (Sigma, St. Louis MO), 1% goat serum]. Test sera were diluted 1/25 followed by serious 3-fold dilutions in blocking buffer. The block solution was aspirated the plates were incubated at 37° for 2 hours with 150µl/well of Goat anti-mouse IgG-HRP (Caltag, Burlingame, CA) diluted 1/40,000 in block buffer. Following a final 6 washes, the plates were developed with OPD for 30 min. The OPD developer consists of 1 tablet (10 mg) o-phenylenediamine, 12 ml buffer (0.1M citric acid, 0.1M dibasic sodium phosphate), 5µl 30% H_2O_2. The reaction was stopped with 50µl per well 4H H_2SO_4 and optical density was measured at dual wavelengths 492-690. The reported titers correspond to the reciprocal of the serum dilution producing an absorbance value of 1.0.

Example 2
Enhancement Of Luciferase Gene Expression In Muscle Cells In Mammals

Previous reports have demonstrated that application of electric current after injection of plasmid DNA has resulted in increased expression of the encoded proteins in the injected tissues (for example see Mir *et al.* (1999) *CR Acad. Sci. III 321*:893-899; Mathieson (1999) *Gene Ther. 6*: 508-514). In order to demonstrate the ability of electoporation and iontophoresis to facilitate the distribution and/or uptake of DNA into mammalian cells and tissue, mice were injected with DNA encoding the readily discernable marker enzyme luciferase (Luc).

Immunization Procedure

Female 6-8 week old CB6F1 mice (Charles River) were anesthetized using 4 parts ketamine HCl, 100 mg/ml stock solution (Fort Dodge Animal Health, Fort Dodge, Iowa), 1 part xylazine, 20 mg/ml (Lloyd Labs, Shenandoah, Iowa). The mice received 1 µl per mg of body weight intramuscularly in the posterior thigh. The tibialis anterior (TA) muscle was shaved and the animals were injected with 10 µg of plasmid in a volume of 50 µl. To control needle depth, a 0.3 cc insulin syringe was covered with polyethylene tubing (i.d. 0.38) to expose only the bevel.

In some instances, electric current was applied to the injected muscles as follows. For constant current deliveries (iontophoresis), plasmid DNA in 5% dextrose was injected into the right tibialis anterior muscle using a single needle delivery probe, which has a functional length of 3 mm. Following plasmid injection, the plasmid delivery needle was attached to the negative lead from the controller and a needle electrode placed in the contralateral leg was attached to the positive lead. Constant current pulses of 5 mA in amplitude, 10 msec in width, were given at a frequency of 1 Hz for 1 min. For constant voltage deliveries (electroporation), plasmid DNA in PBS was injected into the right tibialis anterior muscle as previously described. Electrical energy delivery was performed through a bipolar needle probe that was placed over the site of plasmid injection. The probe needles had a separation distance of 0.4 cm and a needle length of 0.3 cm.

The probe was connected to a constant voltage power supply and 5 constant voltage pulses, 50 msec in width, either 100 or 200 V cm-1, were applied in one orientation, the probe was rotated 90 degrees and 5 additional pulses were applied.

Measurement of Luciferase Activity

Mice were sacrificed up to 14 days post vaccination, and TA muscles were collected and flash frozen in liquid nitrogen. The frozen tissue was homogenized with a mortar and pestle (on dry ice), lysed with 0.5 ml 1X reagent lysis buffer (Promega, Madison, WI), and vortexed for 15 minutes at room temperature. The samples were subjected to 3 freeze thaws and centrifuged for 10 minutes at 10,000 X g. Supernatants were collected and stored at -80°C until assayed. The ML3000 microplate luminometer (Dynex Technologies, Chantilly, VA) measured the luciferase activity by automatically dispensing 100 µl of luciferase assay reagent (Promega, Madison, WI) into wells containing 20 µl of supernatant, and measuring the relative light units (RLU). The setting for the luminometer were the following, Mode: enhanced flash, Gain: medium, Delay time: 1 sec., Integrate time: 5 sec., calibrate each run. Sample values were extrapolated from a standard curve prepared from QuantiLum® Recombinant Luciferase (Promega, Madison, WI). Results are expressed as ng luciferase per mg muscle protein, with protein determination by BCA Protein Assay Reagent (Pierce).

The results of this experiment are shown in **Table 1**, and indicate that electoporation and iontophoresis facilitated the distribution and/or uptake of DNA into mammalian cells and tissue. In **Table 1**, results are expressed as ng luciferase activity per mg muscle protein. Numbers in parentheses indicate standard deviation of the mean (sd).

Table 1			
Luc DNA Treatment	**Luc Activity**	**Mean (sd)**	**Fold Increase**
(control) (10 µg)	6.76 0.74 0.44 9.11 1.92	3.794 (3.91)	1.00
Ionto (10 µg)	26.63 10.54 23.46 5.51 20.61	17.35 (8.95)	4.57
Electro (10 µg)	18.5 35.02 39.02 33.22 13.06	27.764 (11.30)	7.32

Example 3
Enhancement Of Luciferase Gene Expression In Muscle Cells In Mammals

In order to assess the duration of luciferase gene expression in mammalian tissue, groups of 6 CB6 F1 mice were inoculated with 10 µg of luciferase (Luc) DNA in the TA muscle of one leg. One group of mice was not further treated and one group was treated with electroporation (Electro). At 4 and 14 days after inoculation, the muscles were collected and luciferase activity was measured and expressed as ng luciferase activity per mg muscle protein. The data (**Table 2**) showed a significant enhancement of luciferase gene expression in mammalian tissue that had been subjected to electroporation, relative to non-electroporated, control animals. In **Table 2**, numbers in brackets indicate standard deviation of the mean (sd).

Table 2

Luc DNA Treatment	4 Days		14 Days	
	Luc activity	Mean (sd)	Luc activity	Mean (sd)
(control) (25 µg)	0 5.02 0.06 0.17 0.05 0	1.32 (2.03)	0 0 0 0 0 0	0 (0)
(Electro) (10 µg)	17.3 42.9 9.38 69.7 92.8 72.9	50.8 (33.2)	5.26 3.63 18.9 7.52 40.4 0.71	12.7 (14.9)

Example 4
Enhancement Of β-Galactosidase Gene Expression In Muscle Cells In Mammals

To further demonstrate the ability of electoporation and iontophoresis to facilitate the distribution and/or uptake of DNA into mammalian cells and tissue, mice were injected with DNA encoding a different readily discernable marker enzyme (β-galactosidase).

CB6 F1 mice were inoculated with 100 µg of pCMV β-gal, a β-galactosidase-encoding DNA, in the TA muscle of one leg. The plasmid uses the same promoter as that used for HIV gag and env to express β-galactosidase. One group of mice was not further treated, one group was treated with electroporation, and another with iontophoresis. At 1 day after inoculation, the muscles were collected and prepared for microscopy (magnification = X). The data (**Figure 1A** (untreated); **Figure 1B** (electroporation)) indicated that electroporation had substantially facilitated the distribution and/or uptake of DNA into mammalian cells and tissue. A similar result was observed in mouse tissue that had been subjected to iontophoresis.

Thus, DNA plasmids encoding the reporter genes luciferase and β-galactosidase were employed to measure transfection of muscles cells *in vivo*. At 4 and 14 days after a single inoculation of DNA, luciferase expression was found to be higher in muscles treated with electric current (as compared to untreated muscles (see **Example 2, Table 1**)). This was true for muscles that had been subjected to both iontophoresis (4.6-fold) and electroporation (7.3-fold). Similarly, the number of muscle fibers detectably transfected after inoculation of β-galactosidase DNA was found to have been substantially increased by iontophoresis and electroporation, as compared to untreated muscles, as judged by β-galactosidase staining of muscle tissue sections. In addition, as noted previously (Mir *et al.* (1999) *CR Acad. Sci. III 321*:893-899), application of electric current appears to decrease the variability of reporter gene expression in muscle cells. Therefore, application of electric current facilitates delivery of DNA to muscle cells *in situ* promotes efficient transfection.

Example 5
Enhancement Of Antibody Responses In Mammals

In order to demonstrate the ability of electroporation and iontophoresis to enhance the antibody responses of mammals, groups of 4-6 CB6 F1 mice were inoculated a single time with 10 μg of DNA encoding the HIV gag protein.

The plasmid pCMV HIV p55 gag, grown in E. coli strain HB101, as described above, was employed as the source of the gag-encoding DNA. The DNA was inoculated into the TA muscle of one leg. One group of mice was not further treated, one group was treated with iontophoresis and another with electroporation. Sera from mice were analyzed for anti-gag antibody titer at 2, 4, 8 and 12 weeks after inoculation. The data are shown in **Figure 2**. In **Figure 2**, data are plotted as geometric mean ELISA titer and error bars indicate SEM. At all time points tested, antibody titers in mice that had been subjected to iontophoresis and electroporation were 8- to 20-fold higher than in animals receiving no further treatment (**Figure 2**). As with luciferase expression levels, in general, electroporation conditions

appeared slightly superior to iontophoresis for enhancement of antibody responses. The data indicate a pronounced enhancement of antibody response in animals subjected to electroporation and iontophoresis, relative to the response of control animals.

Example 6
Effect Of Vaccine Boosting On Antibody Responses In Mammals

In order to demonstrate the effect of vaccine boosting on antibody responses in mammals, groups of 6 CB6 F1 mice were inoculated with 10 μg of DNA encoding the HIV gag protein. Inoculation was into the TA muscle of one leg of the animals at 3 and 6 weeks. One group of mice was not further treated (**Figure 3**, open bars), one group was treated with iontophoresis (**Figure 3**, solid bars) and another with electroporation (**Figure 3**, shaded bars). Sera were collected at 3 weeks after each immunization and analyzed for antibody responses. Data are plotted as geometric mean ELISA titer and error bars indicate SEM. Antibody titers were elevated in all groups after the booster injection, but the approximately 10-fold enhancement in titers observed in mice receiving electric current was maintained even after the boost (**Figure 3**).

Example 7
Effect Of Conditions Of Iontophoresis And Electrophoresis On Antibody Responses In Mammals

In order to demonstrate the effect of the conditions of iontophoresis and electroporation on mammalian antibody responses, groups of 6 CB6 F1 mice were inoculated with 10 μg of HIV gag DNA (obtained as described above) in the TA muscle of one leg at 3 weeks. Groups of mice were treated as indicated in **Table 3**. Sera were collected at 3 weeks and analyzed for antibody responses. In **Table 3**, data are tabulated as geometric mean ELISA titer and as fold increase over titers achieved in vaccinated but untreated mice. The results show that enhancement of DNA vaccine potency is achieved across a wide range of conditions.

Table 3					
Treatment	**Conditions**	**Number of Pulses**	**Duration (msec)**	**Geometric Mean Titer**	**Fold Increase**
DNA control	-	-	-	414	1
Ionto	50 mA	60	10	1071	2.59
Ionto	50 mA	10	10	2521	6.09
Ionto	50 mA	10	50	1738	4.20
Ionto	100 mA	10	10	1876	4.53
Ionto	100 mA	10	50	2293	5.54
Electro	25 V/cm	10	10	479	1.16
Electro	50 V/cm	10	10	1099	2.65
Electro	50 V/cm	10	50	2390	5.77
Electro	100 V/cm	10	50	1800	4.35
Electro	200 V/cm	10	10	2208	5.33
Electro	200 V/cm	10	50	2079	5.02
Electro	300 V/cm	10	10	2834	6.85
Electro	400 V/cm	10	10	1359	3.28

Example 8
Efficacy Of Intradermal Administration
Of Iontophoresis And Electroporation In Mammals.

In order to assess the efficacy of intradermal administration of iontophoresis and electroporation in mammals, groups of 6 CB6 F1 mice were inoculated with 10 μg of HIV gag DNA intradermally on the backs. One group of mice was not further treated (DNA control), one group was treated with iontophoresis and another with electroporation at the conditions indicated in **Table 4**. Sera were collected at 3 weeks after immunization and analyzed for antibody responses. In **Table 4**, data are tabulated as geometric mean ELISA titer and fold increase over titers achieved in vaccinated but untreated mice. As shown, electroporation and iontophoresis are also effective for the intradermal route of administration of DNA vaccines.

Table 4					
Treatment	**Conditions**	**Number of Pulses**	**Duration (msec)**	**Geometric Mean**	**Fold Increase**
DNA control	-	-	-	472	1
Ionto	50 mA	60	10	696	1.47
Ionto	50 mA	10	10	1291	2.74
Ionto	50 mA	10	50	626	1.33
Ionto	100 mA	10	10	2376	5.03
Ionto	100 mA	10	50	1134	2.40
Electro	150 V/cm	10	10	2768	5.86
Electro	300 V/cm	10	10	851	1.80
Electro	450 V/cm	10	10	132	0.28
Electro	600 V/cm	10	10	887	1.88
Electro	750 V/cm	10	10	480	1.02
Electro	75 V/cm	10	50	224	0.47
Electro	150 V/cm	10	50	728	1.54
Electro	225 V/cm	10	50	2202	4.67
Electro	300 V/cm	10	50	6125	12.98
Electro	375 V/cm	10	50	937	1.99

Example 9
Efficacy Of Plate Electrode For
Iontophoresis And Electroporation In Mammals

In order to demonstrate the efficacy of employing a plate electrode for iontophoresis and electroporation in mammals, groups of 6 CB6 F1 mice were inoculated with 10 μg of HIV gag DNA in the TA muscle of one leg. Groups of mice were treated as indicated in **Table 5**. The combination needle and plate electrode system consists of 3 electrically conducting components, plus electrical leads for connections, and a holder apparatus. Two of the electrically conductive components represent needle electrodes, of the same polarity (typically negative). These needle electrodes are fabricated of stainless steel (cylindrical, grade 316). Needle lengths were 3mm. The needles were encapsulated within insulation, and were retained in the electrode assembly, surrounded by the plate electrode. The plate electrode consisted of a stainless steel block, with dimensions of 1 x 1 x 1 cm. The needle electrodes extended through the plate electrode, with approximately 3 mm length extending beyond the surface of the electrode. Insulation around the needle prevented passage of electric current from the needle

directly to the plate electrode. For in vivo application, the electric current path was from the power source through the connector cable to the needle electrodes. Electric current was then transmitted from the end of the needle electrodes through biological tissue, to the plate electrode, and thus through a connecting cable to the power source, completing the circuit. The shortest electrically conductive path through tissue is approximately 2.5 mm. This is accounted for by the 2 mm of insulated needle electrode extending above the plate electrode, and the diameter of the holes through the plate electrode, through which the needle electrodes extend.

The electrode assembly was used to deliver a series of electrical energy pulses in either constant voltage (electroporation) or a constant current (iontophoresis) mode. Sera were collected at 6 weeks and analyzed for antibody responses. One group of mice was not further treated (DNA control). Other groups were treated with iontophoresis and electroporation at the indicated conditions. In **Table 5**, data are tabulated as geometric mean ELISA titer and fold increase over titers achieved in vaccinated but untreated mice. The results indicate that a significant increase in antibody titer could be obtained using the needle and plate electrode system to deliver current for electroporation or iontophoresis.

Table 5

Treatment	Conditions	Number of Pulses	Duration (msec)	Geometric Mean	Fold Increase
DNA control	-	-	-	198	1
Ionto	50 mA	10	10	1596	8.06
Ionto	100 mA	10	10	1235	6.24
Electro	200 V/cm	10	10	1411	7.13
Electro	300 V/cm	10	10	1252	6.32

Example 10
Efficacy Of Electroporation On Anti-HIV Gag Antibody Responses in Mammals

In order to demonstrate the efficacy of electroporation on the anti-HIV gag antibody response of mammals, groups of 4-6 New Zealand white rabbits were inoculated with a combination DNA vaccine consisting of 500 μg of DNA encoding the HIV gag protein and 1 mg of DNA encoding the HIV env protein.

The plasmid pCMV HIV gag was used as the source of the gag- encoding DNA. Plasmid pCMV HIV env was employed as the source of the env-encoding DNA. The plasmid expresses high levels of HIV-1 env, due to a potent CMV promoter with intron A and a codon-optimized env-encoding region (see U.S. Provisional Patent Application Serial No. 60/168,471, filed December 1, 1999).

Inoculations were into the hind leg gracilis muscles at 0, 6 and 12 weeks. One group of rabbits received DNA without further treatment (DNA control). Other groups were treated with electroporation with a 6-needle electrode or a 2-needle electrode. The two-needle array electrodes (BTX) were inserted into the muscle immediately after DNA delivery for electroporation. The distance between the electrodes was 5 mm and the array was inserted longitudinally relative to the muscle fibers. *In vivo* electroporation parameters were: 20V/mm distance between the electrodes, 50 msec pulse length, 6 pulses with reversal of polarity after three pulses, at 1 pulse per second, given by a BTX 820 square wave generator. The electroporation with a 6-needle electrode array formed a circle (Genetronics, Inc.). The diameter of the electrode array was 1 cm, with a needle length of 1 cm. Six electroporation pulses of 20V/mm, 50 msec pulse length, one pulse per second were given by a BTX 820 square wave generator, combined with an electronic switch (Genetronics, Inc.) to rotate the electric field in 60 degree increments after each discharge (Hofmann *et al.* (1996) *IEEE Engineer. Med. Biol. 15*:124-132).

At 26 weeks, all rabbits were boosted with recombinant gag protein and sera were collected at 2 weeks post-protein boost and analyzed for anti-gag antibody responses. Anti-HIV gag antibodies were measured by ELISA as follows. Wells of Immulon 2 HB "U" bottom microtiter plates (Dynex Technologies, Chantilly, VA) were coated with HIV p55 protein at 5μg/ml in PBS, 50 μl per well, and incubated at 4°C overnight. The plates were washed 6X with wash buffer [PBS, 0.1% Tween 20 (Sigma, St. Louis, MO)] and blocked at 37°C for 1 hour with 150μl/well blocking buffer [PBS, 0.5% casein, and 5% goat serum]; the dilution buffer was blocking buffer plus 0.3% Tween 20. The secondary antibody was goat anti-rabbit IgG used at 1/20,000; and the OD cutoff used was 0.6. Test

sera were diluted 1/25 followed by serial 3-fold dilutions in blocking buffer. The blocking buffer was aspirated and the plates were incubated at 37°C for 2 hours with 50µl/well of each dilution. After washing 6 times, the plates were incubated for 1 hour at 37°C with 50µl/well of Goat anti-mouse IgG-HRP (Caltag, Burlingame, CA) diluted 1/40,000 in blocking buffer. Following a final 6 washes, the plates were developed with OPD for 30min. The OPD developer consists of 1 tablet (10 mg) o-phenylenediamine, 12 ml buffer (0.1M citric acid, 0.1M dibasic sodium phosphate), 5µl 30% H_2O_2. The reaction was stopped with 50µl per well 4N H_2SO_4 and optical density was measured at dual wavelengths 492-690. The reported titers correspond to the reciprocal of the serum dilution producing an absorbance value of 1.0.

For measurement of anti-env antibodies in rabbits and guinea pigs, Nunc Immunoplate U96 Maxisorp plates (Nalge Nunc International, Rochester, NY) were coated with 200ng per well of recombinant gp120SF2 protein and incubated for at least 14 hours at 4°C. Between steps, the plates were washed in a buffer containing 137mM NaCl and 0.05% Triton X100. Serum samples were initially diluted 1:25 or 1:100 (in a buffer containing 100mM $NaPO_4$, 0.1% Casein, 1mM EDTA, 1% Triton X-100, 0.5M NaCl and 0.01% Thimerosal, pH 7.5) and were serially diluted 3-fold. The plates were incubated for 50 minutes. After washing in a buffer containing 137mM NaCl, 0.05% Triton X-100, the samples were then reacted with an HRP-conjugated second antibody. The plates were then developed using a TMB substrate kit (Pierce, Rockford, IL). The plates were stopped with either 2N H_2SO_4 or 10% SDS, respectively and read at wavelengths of 450nm or 415nm, respectively. Anti-env antibody responses were measured as the dilution at which an OD of 0.6 was achieved.

The data is shown in **Figure 4**, and indicates that electroporation was effective in enhancing the induced immune response. In **Figure 4**, data are plotted as geometric mean ELISA titer and error bars indicate SEM.

Example 11
Efficacy Of Electroporation On Anti-HIV Env Antibody Responses in Mammals

As a further demonstration of the efficacy of electroporation on the antibody response of mammals, groups of 4 New Zealand white rabbits were inoculated with a combination DNA vaccine consisting of 500 μg of HIV gag-encoding DNA and 1 mg of HIV env-encoding DNA (obtained as described above) in the hind leg muscles at 0 and 6 weeks. One group of rabbits received DNA without further treatment and one group was treated with electroporation with a 6-needle electrode as described above. Sera were collected at 2 weeks post the second DNA immunization and analyzed for anti-env antibody responses. The data are shown in **Table 6**. In **Table 6**, data are tabulated as individual ELISA titers, geometric mean ELISA titers (GMT) and fold increase over titers achieved in vaccinated but untreated rabbits. The data show a pronounced enhancement of antibody titer in animals subjected to electroporation.

Table 6

Conditions	Titer	Geometric Mean Titer	Fold Increase
DNA control	36 8 141 86	44	1
6-needle Electro	2660 762 821 289	833	18.93

It will be apparent to those skilled in the art that various modifications may be made in the present invention without departing from the spirit and scope of the present invention. It will be additionally apparent to those skilled in the art that the basic construction of the present invention is intended to cover any variations, uses or adaptations of the invention following, in general, the principle of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains. Therefore, it will

be appreciated that the scope of this invention is to be defined by the claims appended hereto, rather than the specific embodiments which have been presented as examples. All references and documents cited herein are incorporated by reference herein in their entirety.

What Is Claimed Is:

1. A method of enhancing an immune response generated in an animal comprising the steps of:

(A) administering to the animal DNA encoding one or more immunogen of interest; and

(B) applying an electric field to at least the site of such DNA administration.

2. The method of claim 1, wherein said immune response comprises enhanced production of antibody specifically reactive with said immunogen.

3. The method of claim 1, wherein said immune response comprises enhanced production of lymphocytes that produce antibody specifically reactive with said immunogen.

4. The method of claim 1, wherein said animal is a mammal.

5. The method of claim 4, wherein said mammal is selected from the group consisting of a cat, a dog, a horse, a human, a rabbit and a rodent.

6. The method of claim 5, wherein said mammal is a human.

7. The method of claim 1, wherein said immunogen is a protein or peptide of a pathogen.

8. The method of claim 7, wherein said pathogen is selected from the group consisting of a bacterium, a fungus, a yeast, a protozoan, and a virus.

9. The method of claim 8, wherein said pathogen is a bacterium selected from the group consisting of an enteric bacterium, a *Clostridrium*, a *Vibrio*, a *Nocardia, a Corynebacterium*, a *Listeria*, a *Legionella,* a *Bacilli*, a

Staphylococcus, a *Streptococci*, a *Borrelia*, a *Mycobacterium*, a *Neisserium* and a *Trepanoma* bacterium.

10. The method of claim 8, wherein said pathogen is a fungus selected from the group consisting of a *Dermatophyte*, a *Pneumocystis*, a *Trypanosoma*, a *Plasmodium*, a *Candida*, a *Cryptococcus*, a *Histoplasma*, a *Coccidioide*, an *Amoeba* and a Schistosome.

11. The method of claim 8, wherein said pathogen is a virus selected from the group consisting of a parvovirus, an orthomyxovirus, a paramyxovirus, a picornavirus, a papovirus, a herpesvirus, a togavirus, and a retrovirus.

12. The method of claim 11, wherein said pathogen is the retrovirus HIV.

13. The method of claim 12, wherein the DNA administered in step (A) encodes one or more HIV protein or peptide.

14. The method of claim 13, wherein said HIV protein or peptide is the HIV gag protein or a peptide fragment thereof.

15. The method of claim 14, wherein said DNA administered in step (A) comprises a codon-optimized gag-encoding region.

16. The method of claim 13, wherein said HIV protein or peptide is the HIV env protein or a peptide fragment thereof.

17. The method of claim 16, wherein said DNA administered in step (A) comprises a codon-optimized env-encoding region.

18. The method of claim 13, wherein said DNA administered in step (A) encodes both (a) an HIV gag protein or a peptide fragment thereof and (b) an HIV env protein or a peptide fragment thereof.

19. The method of claim 18, wherein said DNA administered in step (A) comprises a codon-optimized gag-encoding region and a codon-optimized env-encoding region

20. The method of claim 1, wherein said DNA encoding one or more immunogen of interest is administered to said animal incorporated in a plasmid form.

21. The method of claim 1, wherein said DNA encoding one or more immunogen of interest is administered to said animal associated with protein or lipid.

22. The method of claim 1, wherein said DNA is administered to said animal by intramuscular or intradermal injection.

23. The method of claim 1, wherein in step (B) said electrical field is applied under electroporation conditions.

24. The method of claim 1, wherein in step (B) said electrical field is applied under iontophoresis conditions.

25. The method of claim 1, wherein said DNA is administered using a device selected from the group consisting of a single needle probe, a bipolar probe and a combination needle and plate probe.

26. An apparatus for enhancing an immune response in an animal comprising:

(A) DNA encoding one or more immunogen of interest;

(B) means for administering said DNA to said animal; and

(C) means for applying an electric field to at least the site of such DNA administration.

27. The apparatus method of claim 26, wherein said immunogen is a protein or peptide of a pathogen.

28. The apparatus of claim 27, wherein said pathogen is selected from the group consisting of a bacterium, a fungus, a yeast, a protozoan, and a virus.

29. The apparatus of claim 28, wherein said pathogen is a bacterium selected from the group consisting of an enteric bacterium, a *Clostridrium*, a *Vibrio*, a *Nocardia, a Corynebacterium*, a *Listeria*, a *Legionella,* a *Bacilli*, a *Staphylococcus*, a *Streptococci*, a *Borrelia*, a *Mycobacterium*, a *Neisserium* and a *Trepanoma* bacterium.

30. The apparatus of claim 28, wherein said pathogen is a fungus selected from the group consisting of a *Dermatophyte,* a *Pneumocystis*, a *Trypanosoma*, a *Plasmodium*, a *Candida,* a *Cryptococcus,* a *Histoplasma,* a *Coccidioide*, an *Amoeba* and a Schistosome.

31. The apparatus of claim 28, wherein said pathogen is a virus selected from the group consisting of a parvovirus, an orthomyxovirus, a paramyxovirus, a picornavirus, a papovirus, a herpesvirus, a togavirus, and a retrovirus.

32. The apparatus of claim 31, wherein said pathogen is the retrovirus HIV.

33. The apparatus of claim 32, wherein the DNA administered in step (A) encodes one or more HIV protein or peptide.

34. The apparatus of claim 33, wherein said HIV protein or peptide is the HIV gag protein or a peptide fragment thereof.

35. The apparatus of claim 34, wherein said DNA administered in step (A) comprises a codon-optimized gag-encoding region.

36. The apparatus of claim 33, wherein said HIV protein or peptide is the HIV env protein or a peptide fragment thereof.

37. The apparatus of claim 36, wherein said DNA administered in step (A) comprises a codon-optimized env-encoding region.

38. encodes both (a) an HIV gag protein or a peptide fragment thereof and (b) an HIV env protein or a peptide fragment thereof.

39. The apparatus of claim 38, wherein said DNA administered in step (A) comprises a codon-optimized gag-encoding region and a codon-optimized env-encoding region.

40. The apparatus of claim 26, wherein said DNA encoding said one or more immunogens of interest is incorporated in a plasmid form.

41. The apparatus of claim 26, wherein said DNA encoding one or more immunogen of interest is associated with protein or lipid.

42. The apparatus of claim 26, wherein said means for administering said DNA to said animal accomplishes intramuscular or intradermal administration of said DNA.

43. The apparatus of claim 26, wherein said electrical field is produced under electroporation conditions.

44. The apparatus of claim 26, wherein said electrical field is produced under iontophoresis conditions.

45. The apparatus of claim 26, wherein said means for administering said DNA is a device selected from the group consisting of a single needle probe, a bipolar probe and a combination needle and plate probe.

Fig. 1A

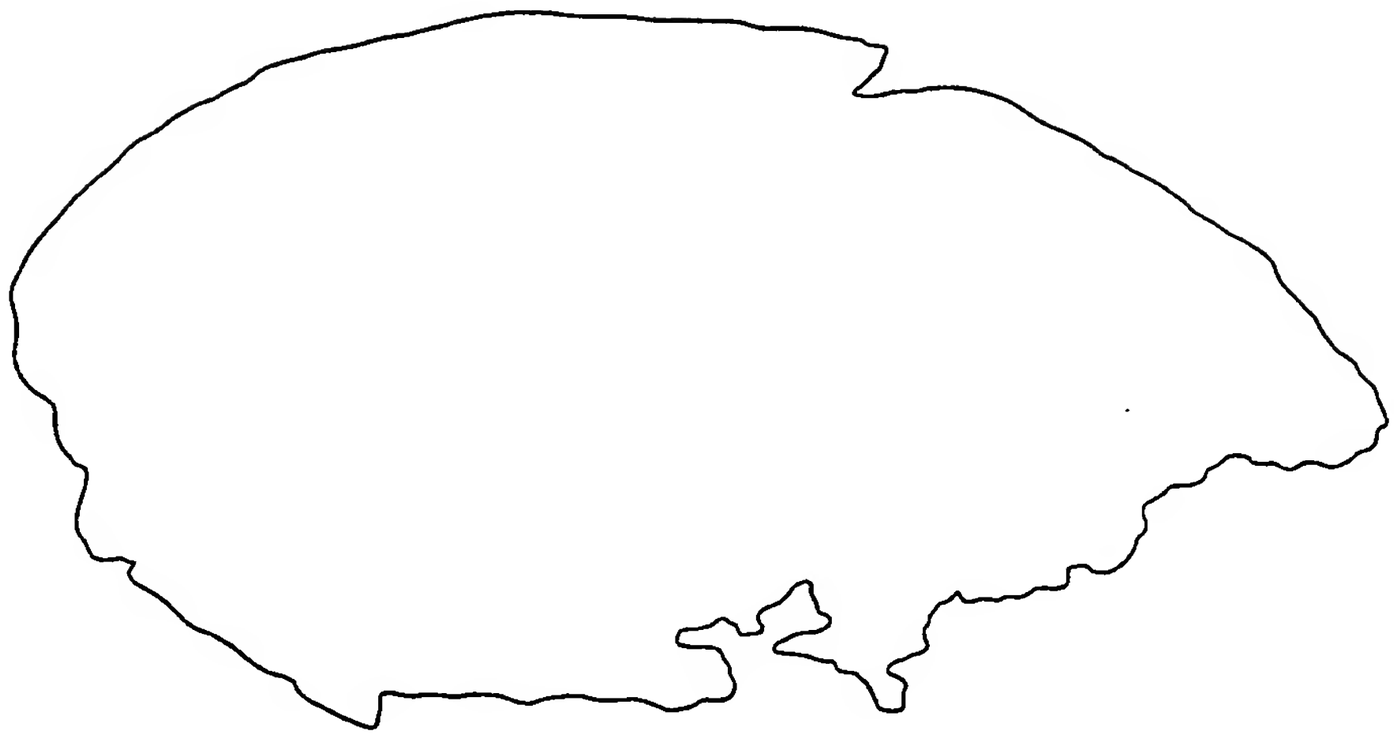


Fig. 1B

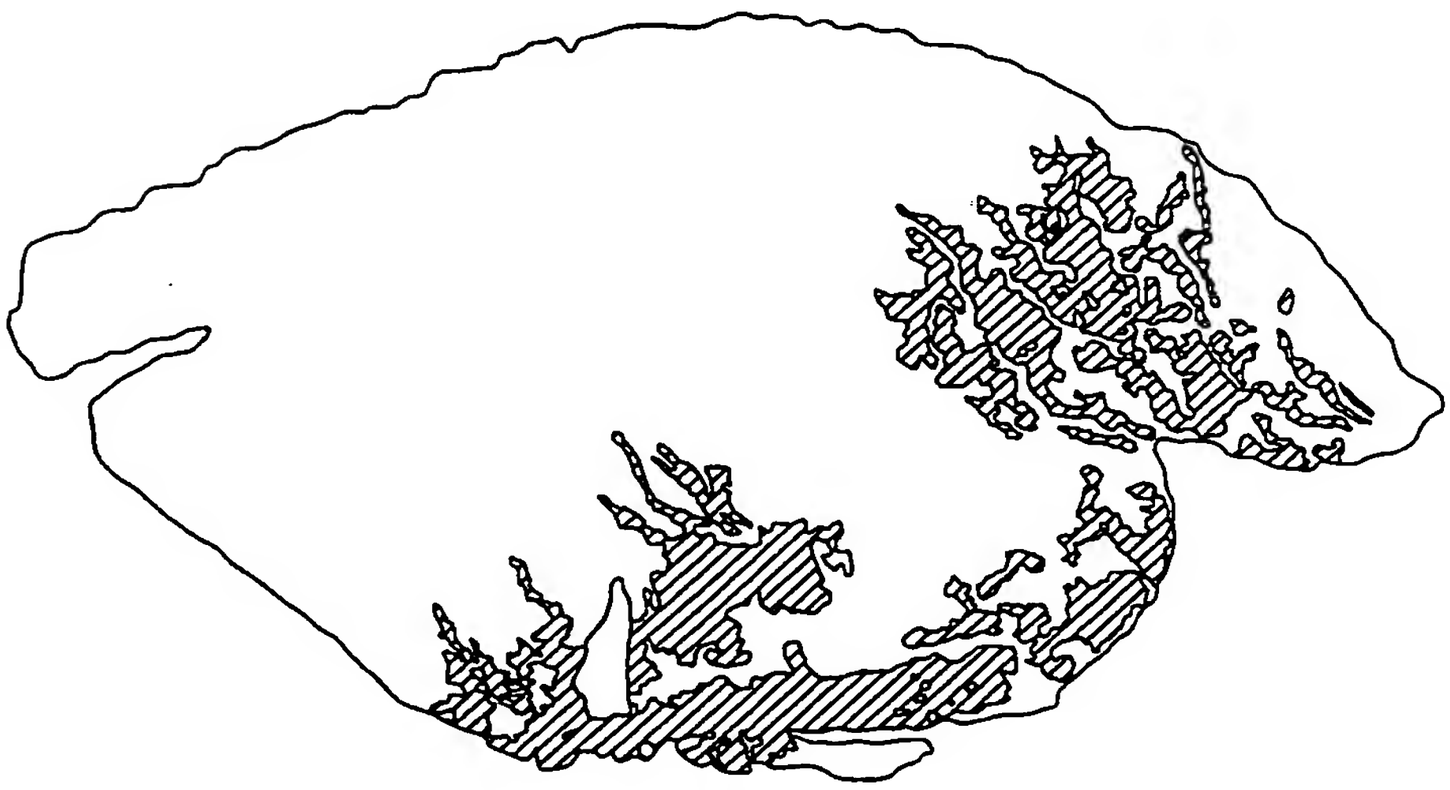


Fig. 2

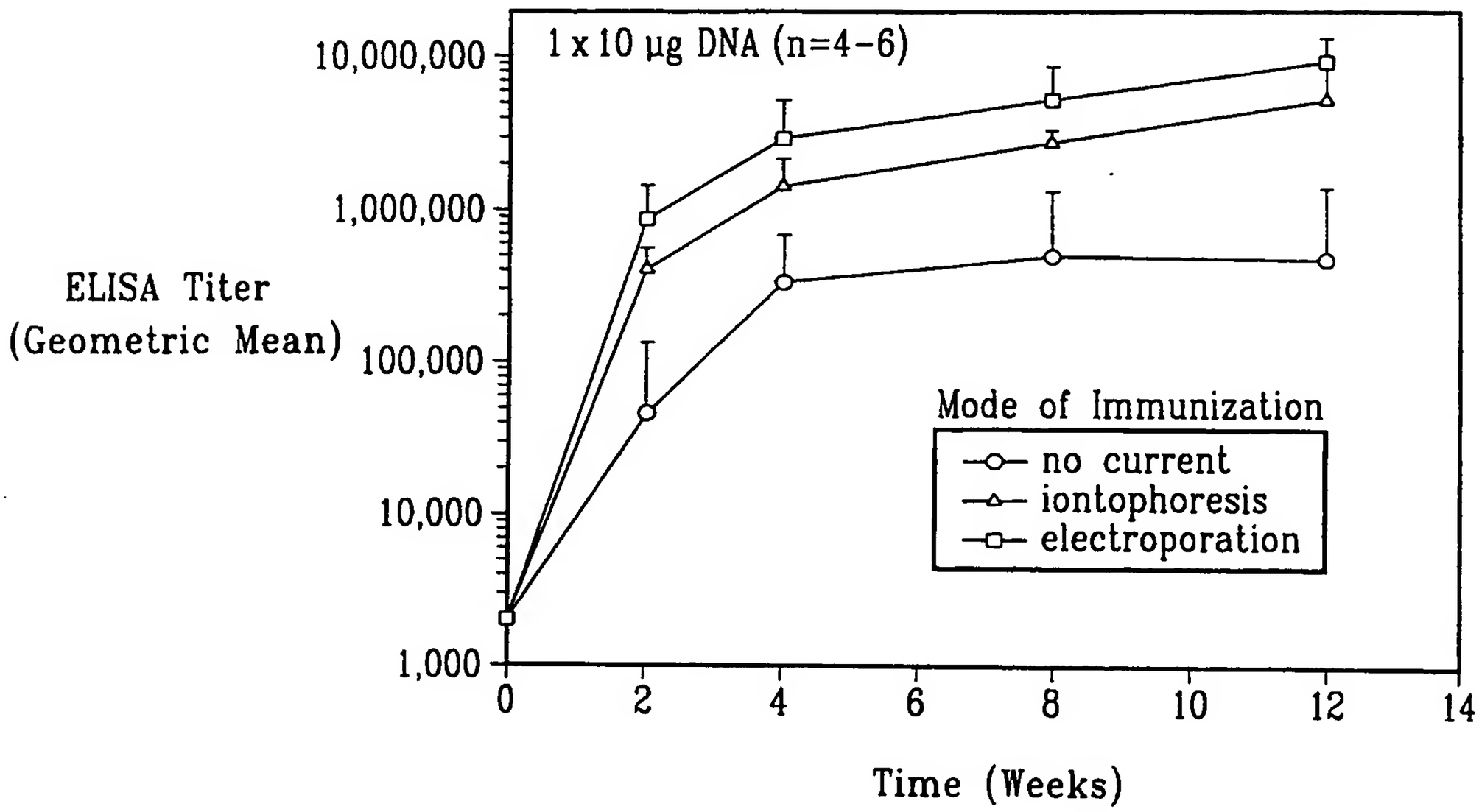


Fig. 3

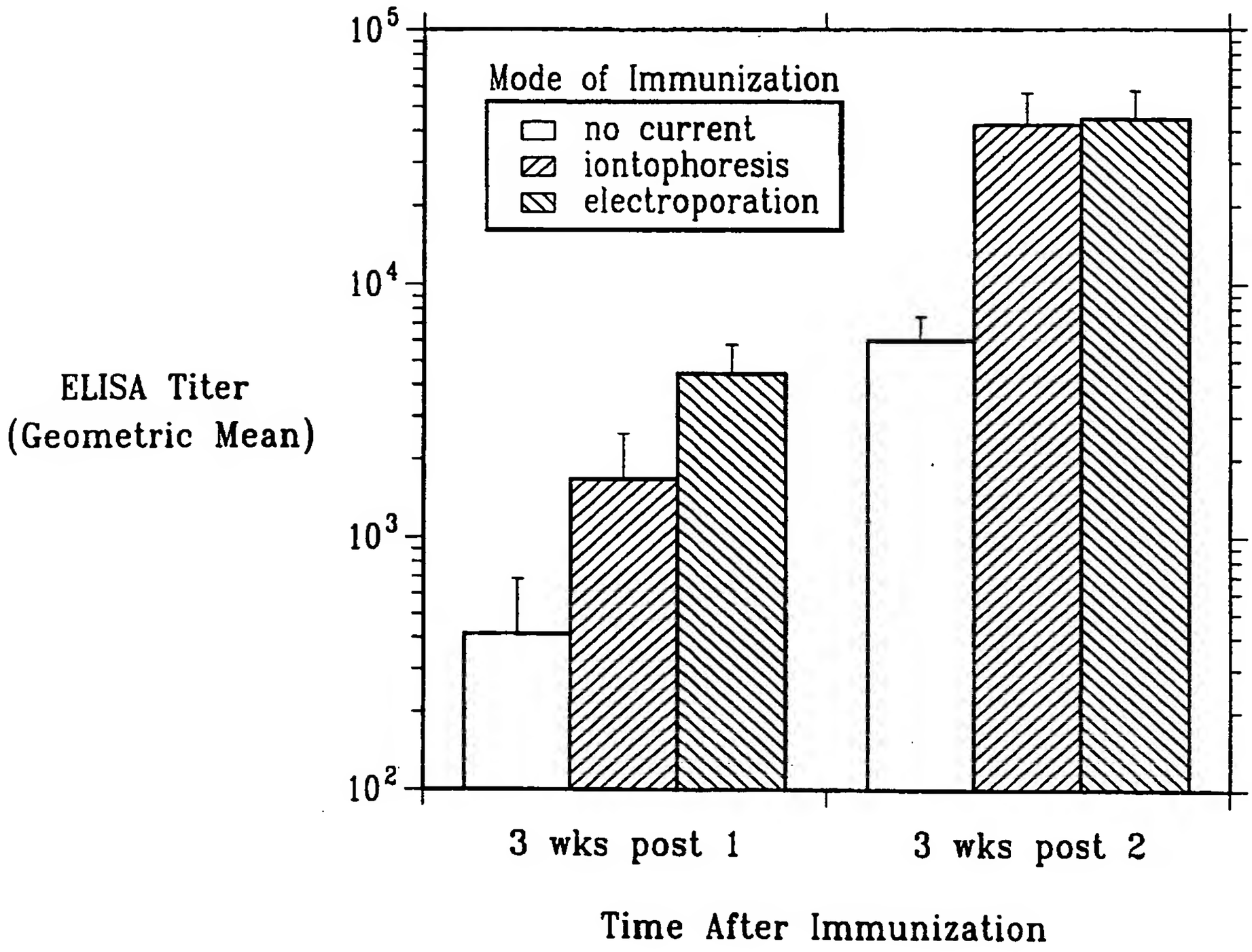
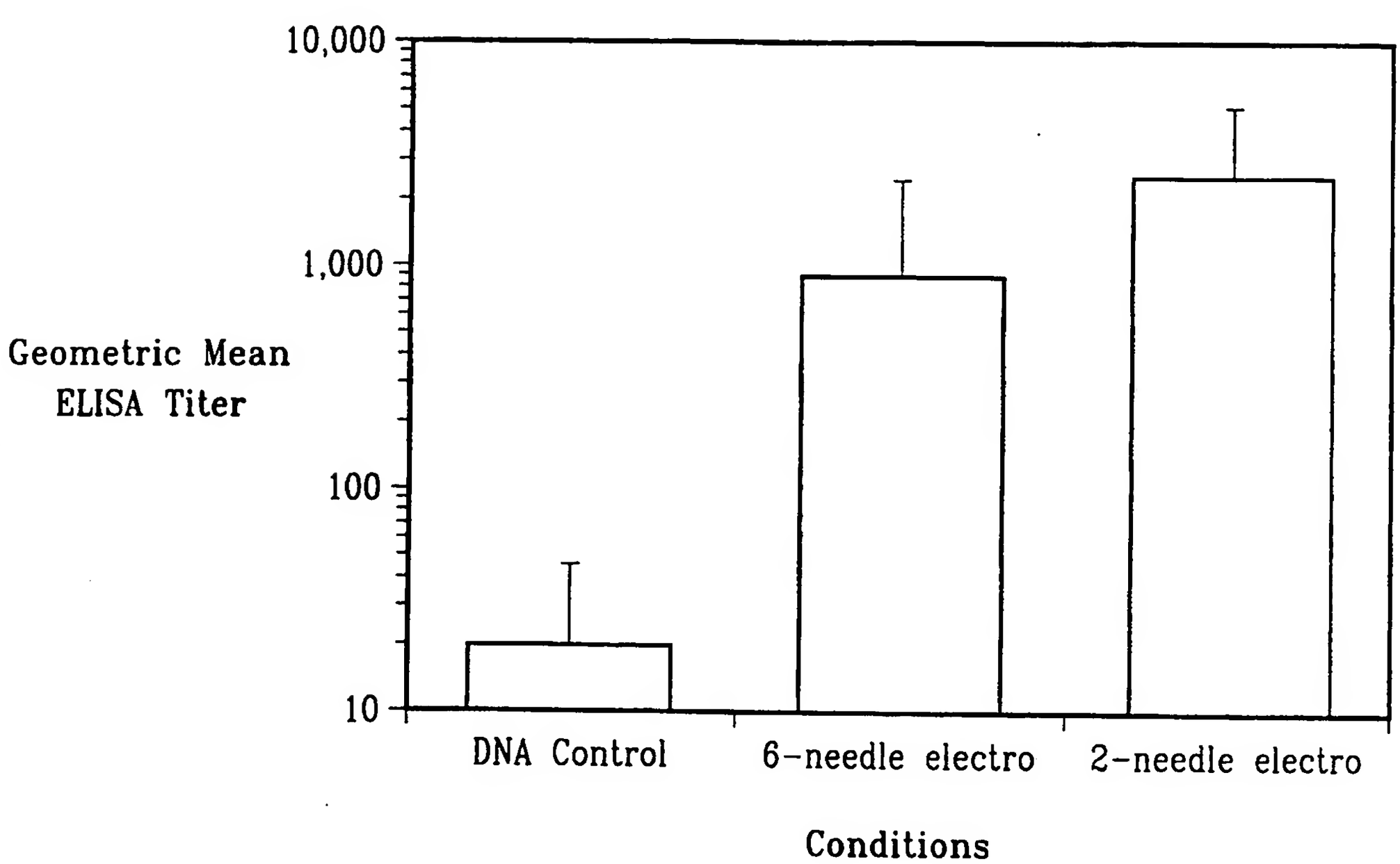


Fig. 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/02831

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/70, 45/00; A61N 1/30, 1/00; C12M 1/42
US CL : 514/44; 424/278.1; 604/20; 607/116; 435/285.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/278.1; 604/20; 607/116; 435/285.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE BIOSIS EMBASE CAPLUS BIOTECHDS WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	NOMURA et al. In vivo induction of cytotoxic T lymphocytes specific for a single epitope introduced into an unrelated molecule. Journal of Immunological Methods. 1993, Vol. 193, pages 41-49, see entire document, especially, abstract; Fig. 1, page 42; page 44, column 1, second full paragraph; and Fig. 4, page 46.	1, 4, 5, 7, 8, 11, 20, 22, 23, 26-29, 31, 42, 43 ---------- 2, 3, 6, 9, 10, 12-19, 21, 24, 25, 30, 32-41, 44, 45

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 APRIL 2000	Date of mailing of the international search report 21 JUN 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RICHARD SCHNIZER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/02831

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,830,877 A (CARSON et al.) 03 November 1998, entire document, especially col. 11, lines 3-15; col. 20, lines 24-32; paragraph bridging columns 20 and 21; col. 24, lines 10-18; col. 37, lines 50 and 51; and Fig. 22.	2, 3, 24, 25, 44, and 45
Y	US 5,593,972 A (WEINER et al.) 14 January 1997, see entire document, especially abstract; col. 6, lines 42-49; col. 13, line 5-19; col. 23, lines 10-20; and col. 34, lines 15-26.	5-19, and 27-39
Y	US 5,786,464 A (SEED) 28 July 1998, see entire document, especially col. 1, lines 10-12 and 29-34; col. 2, lines 7-13 and 51-55; and column 41, claims 12-14.	8, 11-19, 27, 31-39
Y	EP 0 438 078 A2 (ROSSI et al.) 24 July 1991, see entire document, especially abstract.	25 and 45